(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
3 May 2001 (03.05.2001)

PCT

(10) International Publication Number
WO 01/30394 A1

(51) International Patent Classification[7]: A61K 48/00, A61P 35/00, C12N 15/11, C07H 21/04

(21) International Application Number: PCT/AU00/01315

(22) International Filing Date: 26 October 2000 (26.10.2000)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
PQ 3676 26 October 1999 (26.10.1999) AU

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States *(regional)*: ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 01/30394 A1

(54) Title: TREATMENT OF CANCER

(57) Abstract: The present invention relates to a method for the treatment of tumours, the method comprising inhibiting angiogenesis in a subject in need thereof characterised in that angiogenesis is inhibited by administering to the subject an agent which inhibits induction of EGR, an agent which decreases expression of EGR or an agent which decreases the nuclear accumulation or activity of EGR. The present invention also relates to a method of screening for agents which inhibits angiogenesis.

Treatment of cancer

FIELD OF THE INVENTION

The present invention relates to compositions and methods for the treatment of cancer.

BACKGROUND OF THE INVENTION

Cancer

Cancer accounted for over half a million deaths in the United States in 1998 alone, or approximately 23 % of all deaths (Landis et al., 1998). Only cardiovascular disease consistently claims more lives (Cotran et al., 1999).

There is growing evidence that the cellular and molecular mechanisms underlying tumour growth involves more than just tumour cell proliferation and migration. Importantly, tumour growth and metastasis are critically dependent upon ongoing angiogenesis, the process of new blood vessel formation (Crystal, 1999). Angiogenesis (also known as neovascularisation) is mediated by the migration and proliferation of vascular endothelial cells that sprout from existing blood vessels to form a growing network of microvessels that supply growing tumours with vital nutrients. Primary solid tumours cannot grow beyond 1-2 mm diameter without active angiogenesis (Harris, 1998).

Human HepG2 hepatocellular carcinoma cells have been used as a model cancer cell line for the assessment of anti-neoplastic drugs (Yang et al., 1997). These cells basally and inducibly express the immediately-early gene and transcriptional regulator, early growth response factor-1 (EGR-1) (Kosaki et al., 1995).

Early Growth Response Protein (EGR-1)

Early growth response factor-1 (EGR-1, also known as Egr-1, NGFI-A, zif268, krox24 and TIS8) is the product of an immediate early gene and a prototypical member of the zinc finger family of transcriptional regulators (Gashler et al., 1995). Egr-1 binds to the promoters of a spectrum of genes implicated in the pathogenesis of atherosclerosis and restenosis. These

include the platelet-derived growth factor (PDGF) A-chain (Khachigian et al., 1995), PDGF-B (Khachigian et al., 1996), transforming growth factor-β_1 (Liu et al, 1996,1998), fibroblast growth factor-2 (FGF-2) (Hu et al., 1994; Biesiada et al., 1996), membrane type 1 matrix metalloproteinase (Haas et al., 1999), tissue factor (Cui et al., 1996) and intercellular adhesion molecule-1 (Malzman et al., 1996). EGR-1 has also been localised to endothelial cells and smooth muscle cells in human atherosclerotic plaques (McCaffrey et al., 2000). Suppression of Egr-1 gene induction using sequence-specific catalytic DNA inhibits intimal thickening in the rat carotid artery following balloon angioplasty (Santiago et al., 1999a).

DNAzymes

In human gene therapy, antisense nucleic acid technology has been one of the major tools of choice to inactivate genes whose expression causes disease and is thus undesirable. The anti-sense approach employs a nucleic acid molecule that is complementary to, and thereby hybridizes with, an mRNA molecule encoding an undesirable gene. Such hybridization leads to the inhibition of gene expression.

Anti-sense technology suffers from certain drawbacks. Anti-sense hybridization results in the formation of a DNA/target mRNA heteroduplex. This heteroduplex serves as a substrate for RNAse H-mediated degradation of the target mRNA component. Here, the DNA anti-sense molecule serves in a passive manner, in that it merely facilitates the required cleavage by endogenous RNAse H enzyme. This dependence on RNAse H confers limitations on the design of anti-sense molecules regarding their chemistry and ability to form stable heteroduplexes with their target mRNA's. Anti-sense DNA molecules also suffer from problems associated with non-specific activity and, at higher concentrations, even toxicity. An example of an alternative mechanism of antisense inhibition of target mRNA expression is steric inhibition of movement of the translational apparatus along the mRNA.

As an alternative to anti-sense molecules, catalytic nucleic acid molecules have shown promise as therapeutic agents for suppressing gene expression, and are widely discussed in the literature (Haseloff (1988); Breaker (1994); Koizumi (1989); Otsuka; Kashani-Sabet (1992); Raillard (1996); and Carmi (1996)). Thus, unlike a conventional anti-sense molecule, a catalytic nucleic acid molecule functions by actually cleaving its target

mRNA molecule instead of merely binding to it. Catalytic nucleic acid molecules can only cleave a target nucleic acid sequence if that target sequence meets certain minimum requirements. The target sequence must be complementary to the hybridizing arms of the catalytic nucleic acid, and the target must contain a specific sequence at the site of cleavage.

Catalytic RNA molecules ("ribozymes") are well documented (Haseloff (1988); Symonds (1992); and Sun (1997)), and have been shown to be capable of cleaving both RNA (Haseloff (1988)) and DNA (Raillard (1996)) molecules. Indeed, the development of in vitro selection and evolution techniques has made it possible to obtain novel ribozymes against a known substrate, using either random variants of a known ribozyme or random-sequence RNA as a starting point (Pan (1992); Tsang (1994); and Breaker (1994)).

Ribozymes, however, are highly susceptible to enzymatic hydrolysis within the cells where they are intended to perform their function. This in turn limits their pharmaceutical applications.

Recently, a new class of catalytic molecules called "DNAzymes" was created (Breaker and Joyce (1995); Santoro (1997)). DNAzymes are single-stranded, and cleave both RNA (Breaker (1994); Santoro (1997)) and DNA (Carmi (1996)). A general model for the DNAzyme has been proposed, and is known as the "10-23" model. DNAzymes following the "10-23" model, also referred to simply as "10-23 DNAzymes", have a catalytic domain of 15 deoxyribonucleotides, flanked by two substrate-recognition domains of seven to nine deoxyribonucleotides each. In vitro analyses show that this type of DNAzyme can effectively cleave its substrate RNA at purine:pyrimidine junctions under physiological conditions (Santoro (1997)).

DNAzymes show promise as therapeutic agents. However, DNAzyme success against a disease caused by the presence of a known mRNA molecule is not predictable. This unpredictability is due, in part, to two factors. First, certain mRNA secondary structures can impede a DNAzyme's ability to bind to and cleave its target mRNA. Second, the uptake of a DNAzyme by cells expressing the target mRNA may not be efficient enough to permit therapeutically meaningful results.

SUMMARY OF THE INVENTION

The present inventors have established that EGR-1 is critical in vascular endothelial cell replication and migration and that DNA-based, sequence-specific catalytic molecules targeting EGR-1 inhibit the growth of malignant cells in culture. These findings show that inhibitors of EGR or related EGR family members are useful in the treatment of tumours and that two separate mechanisms of action may involved. Specifically, inhibitors of EGR family members may inhibit tumour growth indirectly by inhibiting angiogenesis and/or directly by blocking the EGR family member in tumour cells.

When used herein the term "EGR" refers to a member of the EGR family. Members of the EGR family are described in Gashler et al., 1995 and include EGR-1 to EGR-4. It is currently preferred that the EGR family member is EGR-1.

Accordingly, in a first aspect the present invention provides a method for the treatment of a tumour, the method comprising administering to a subject in need thereof an agent which inhibits induction of EGR, an agent which decreases expression of EGR or an agent which decreases the nuclear accumulation or activity of EGR.

In a second aspect, the present invention provides a method for inhibiting the growth or proliferation of a tumour cell, the method comprising contacting a tumour cell with an agent which inhibits induction of EGR, an agent which decreases expression of EGR or an agent which decreases the nuclear accumulation or activity of EGR.

In a third aspect, the present invention provides a tumour cell which has been transformed by introducing into the cell a nucleic acid molecule, the nucleic acid molecule comprising or encoding (i) an agent which inhibits induction of EGR, (ii) an agent which decreases expression of EGR, or (iii) an agent which decreases the nuclear accumulation or activity of EGR.

In a fourth aspect, the present invention provides a method of screening for an agent which inhibits angiogenesis, the method comprising testing a putative agent for the ability to inhibit induction of EGR, decrease expression of EGR or decrease the nuclear accumulation or activity of EGR.

In a preferred embodiment of the present invention the agent is selected from the group consisting of an EGR antisense oligonucleotide, a ribozyme targeted against EGR, a ssDNA targeted against EGR dsDNA such that the ssDNA forms a triplex with the EGR-1 ds DNA, and a DNAzyme targeted against EGR.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Insulin stimulates Egr-1-dependent gene expression in vascular endothelial cells. Growth-arrested bovine aortic endothelial cells previously transfected with pEBS1^{3}foscat using FuGENE6 were incubated with D-glucose (5-30 mM), insulin (100 nM) or FGF-2 (25 ng/ml) as indicated for 24 h prior to preparation of cell lysates. CAT activity was normalized to the concentration of protein in the lysates.

Figure 2. Insulin-induced DNA synthesis in aortic endothelial cells is blocked by antisense oligonucleotides targeting Egr-1. A, Insulin stimulates DNA synthesis. Growth-arrested endothelial cells were incubated with insulin (100 nM or 500 nM) or FBS (2.5%) for 18 h prior to ^{3}H-thymidine pulse for a further 6 h. B, Antisense Egr-1 oligonucleotides inhibit insulin-inducible DNA synthesis. Endothelial cells were incubated with 0.8 μM of either AS2, AS2C or E3 prior to exposure to insulin (500 nM or 1000 nM) for 18 h and ^{3}H-thymidine pulse for 6 h. C, Dose-dependent inhibition of insulin-inducible DNA synthesis. DNA synthesis stimulated by insulin (500 nM) was assessed in endothelial cells incubated with 0.4 μM or 0.8 μM of AS2 or AS2C. TCA-precipitable ^{3}H-thymidine incorporation into DNA was assessed using a β-scintillation counter.

Figure 3. Insulin-inducible DNA synthesis in cultured aortic endothelial cells is MEK/ERK-dependent. Growth quiescent endothelial cells were preincubated for 2 h with either PD98059 (10 μM or 30 μM), SB202190 (100 nM or 500 nM) or wortmannin (300 nM or 1000 nM) prior to the addition of insulin (500 nM) for 18 h and ^{3}H-thymidine pulse. TCA-precipitable ^{3}H-thymidine incorporation into DNA was assessed using a β-scintillation counter.

Figure 4. Wound repair after endothelial injury is potentiated by insulin in an Egr-1-dependent manner. The population of cells in the denuded zone 3 d after injury in the various groups was quantitated and presented histodiagrammatically.

Figure 5. Human microvascular endothelial cell proliferation is inhibited by DNA enzymes targeting human EGR-1. SV40-transformed HMEC-1 cells were grown in MCDB 131 medium with EGF (10 ng/ml) and hydrocortisone (1 μg/ml)

supplements and 10% FBS. Forty-eight hours after incubation in serum-free medium without supplements, the cells were transfected with the indicated DNA enzyme (0.4 μM) and transfected again 72 h after the change of medium, when 10% serum was added. The cells were quantitated by Coulter counter, 24 h after the addition of serum.

Figure 6. Sequence of NGFI-A DNAzyme (ED5), its scrambled control (ED5SCR) and 23 nt synthetic rat substrate. The translational start site is underlined.

Figure 7. NGFI-A DNAzyme inhibits the induction of NGFI-A protein by serum (FBS). Western blot analysis was performed using antibodies to NGFI-A, Sp1 or c-Fos. The Coomassie Blue stained gel demonstrates that uniform amounts of protein were loaded per lane. The sequence of EDC is 5'-CGC CAT TAG GCT AGC TAC AAC GAC CTA GTG AT-3' (SEQ ID NO:1); 3' T is inverted. SFM denotes serum-free medium.

Figure 8. SMC proliferation is inhibited by NGFI-A DNAzyme. **a**, Assessment of total cell numbers by Coulter counter. Growth-arrested SMCs that had been exposed to serum and/or DNAzyme for 3 days were trypsinized followed by quantitation of the suspension. The sequence of AS2 is 5'-CTT GGC CGC TGC CAT-3' (SEQ ID NO:2) . **b**, Proportion of cells incorporating Trypan Blue after exposure to serum and/or DNAzyme. Cells were stained incubated in 0.2% (w:v) Trypan Blue at 22 °C for 5 min prior to quantitation by hemocytometer in a blind manner. **c**, Effect of ED5 on pup SMC proliferation. Growth-arrested WKY12-22 cells exposed to serum and/or DNAzyme for 3 days were resuspended and numbers were quantitated by Coulter counter. Data is representative of 2 independent experiments performed in triplicate. The mean and standard errors of the mean are indicated in the figure. * indicates $P<0.05$ (Student's paired t-test) as compared to control (FBS alone).

Figure 9. NGFI-A DNAzyme inhibition of neointima formation in the rat carotid artery. A neointima was achieved 18 days after permanent ligation of the right common carotid artery. DNAzyme (500 μg) or vehicle alone was applied adventitially at the time of ligation and again after 3 days. Sequence-specific inhibition of neointima formation. Neointimal and medial areas of 5 consecutive

sections per rat (5 rats per group) taken at 250 μm intervals from the point of ligation were determined digitally and expressed as a ratio per group. The mean and standard errors of the mean are indicated by the ordinate axis. * denotes $P<0.05$ as compared to the Lig, Lig+Veh or Lig+Veh+ED5SCR groups using the Wilcoxen rank sum test for unpaired data. Lig denotes ligation, Veh denotes vehicle.

Figure 10. HepG2 cell proliferation is inhibited by 0.75μM of DNAzyme DzA. Assessment of total cell numbers by Coulter counter. Growth-arrested cells that had been exposed to serum and/or DNAzyme for 3 days were trypsinized followed by quantitation of the suspension. The sequence of DzA is 5'-caggggacaGGCTAGCTACAACGAcgttgcggg (SEQ ID NO:3).

DETAILED DESCRIPTION OF THE INVENTION

In a first aspect the present invention provides a method for the treatment of a tumour, the method comprising administering to a subject in need thereof an agent which inhibits induction of an EGR, an agent which decreases expression of an EGR or an agent which decreases the nuclear accumulation or activity of an EGR.

The method of the first aspect may involve indiract inhibition of tumour growth by inhibiting angiogenesis and/or direct inhibition by blocking EGR in tumour cells.

In a preferred embodiment of the first aspect, the tumour is a solid tumour. The tumour may be selected from, without being limited to, a prostate tumour, a hepatocellular carcinoma, a skin carcinoma or a breast tumour.

As will be recognised by those skilled in this field there are a number means by which the method of the present invention may be achieved.

In a preferred embodiment of the present invention, the EGR is EGR-1.

In one embodiment, the method is achieved by targeting the EGR gene directly using triple helix (triplex) methods in which a ssDNA molecule can bind to the dsDNA and prevent transcription.

In another embodiment, the method is achieved by inhibiting transcription of the EGR gene using nucleic acid transcriptional decoys. Linear sequences can be designed that form a partial intramolecular duplex which encodes a binding site for a defined transcriptional factor. Evidence suggests that EGR transcription is dependent upon the binding of Sp1, AP1 or serum response factors to the promoter region. It is envisaged that inhibition of this binding of one or more of these transcription factors would inhibit transcription of the EGR gene.

In another embodiment, the method is achieved by inhibiting translation of the EGR mRNA using synthetic antisense DNA molecules that do not act as a substrate for RNase H and act by sterically blocking gene expression.

In another embodiment, the method is achieved by inhibiting translation of the EGR mRNA by destabilising the mRNA using synthetic antisense DNA molecules that act by directing the RNase H-mediated degradation of the EGR mRNA present in the heteroduplex formed between the antisense DNA and mRNA.

In one preferrede embodiment of the present invention, the antisense oligonucleotide has a sequence selected from the group consisting of

(i) ACA CTT TTG TCT GCT (SEQ ID NO:4), and

(ii) CTT GGC CGC TGC CAT (SEQ ID NO:2).

In another embodiment, the method is achieved by inhibiting translation of the EGR mRNA by cleavage of the mRNA by sequence-specific hammerhead ribozymes and derivatives of the hammerhead ribozyme such as the Minizymes or Mini-ribozymes or where the ribozyme is derived from:

(i) the hairpin ribozyme,

(ii) the Tetrahymena Group I intron,

(iii) the Hepatitis Delta Viroid ribozyme or

(iv) the Neurospera ribozyme.

It will be appreciated by those skilled in the art that the composition of the ribozyme may be;

(i) made entirely of RNA,

(ii) made of RNA and DNA bases, or

(iii) made of RNA or DNA and modified bases, sugars and backbones

Within the context of the present invention, the ribozyme may also be either;

(i) entirely synthetic or

(ii) contained within a transcript from a gene delivered within a virus-derived vector, expression plasmid, a synthetic gene, homologously or heterologously integrated into the patients genome or delivered into cells ex vivo, prior to reintroduction of the cells of the patient, using one of the above methods.

In another embodiment, the method is achieved by inhibition of the ability of the EGR gene to bind to its target DNA by expression of an antisense EGR-1 mRNA.

In another embodiment, the method is achieved by inhibition of EGR activity as a transcription factor using transcriptional decoy methods.

In another embodiment, the method is achieved by inhibition of the ability of the EGR gene to bind to its target DNA by drugs that have preference for GC rich sequences. Such drugs include nogalamycin, hedamycin and chromomycin A3 (Chiang et al J. Biol. Chem 1996; 271:23999).

In a preferred embodiment, the method is achieved by cleavage of EGR mRNA by a sequence-specific DNAzyme. In a further preferred embodiment, the DNAzyme comprises

(i) a catalytic domain which cleaves mRNA at a purine:pyrimidine cleavage site;

(ii) a first binding domain contiguous with the 5' end of the catalytic domain; and

(iii) a second binding domain contiguous with the 3' end of the catalytic domain,

wherein the binding domains are sufficiently complementary to two regions immediately flanking a purine:pyrimidine cleavage site within the region of EGR mRNA corresponding to nucleotides 168 to 332 as shown in SEQ ID NO:15, such that the DNAzyme cleaves the EGR mRNA.

As used herein, "DNAzyme" means a DNA molecule that specifically recognizes and cleaves a distinct target nucleic acid sequence, which may be either DNA or RNA.

In a preferred embodiment, the binding domains of the DNAzyme are complementary to the regions immediately flanking the cleavage site. It will be appreciated by those skilled in the art, however, that strict complementarity may not be required for the DNAzyme to bind to and cleave the EGR mRNA.

The binding domain lengths (also referred to herein as "arm lengths") can be of any permutation, and can be the same or different. In a preferred embodiment, the binding domain lengths are at least 6 nucleotides. Preferably, both binding domains have a combined total length of at least 14 nucleotides. Various permutations in the length of the two binding domains, such as 7+7, 8+8 and 9+9, are envisioned.

The catalytic domain of a DNAzyme of the present invention may be any suitable catalytic domain. Examples of suitable catalytic domains are described in *Santoro and Joyce, 1997* and U.S. Patent No. 5,807,718. In a preferred embodiment, the catalytic domain has the nucleotide sequence GGCTAGCTACAACGA (SEQ ID NO:5).

Within the context of the present invention, preferred cleavage sites within the region of EGR mRNA corresponding to nucleotides 168 to 332 are as follows:

(i) the GU site corresponding to nucleotides 198-199;

(ii) the GU site corresponding to nucleotides 200-201;
(iii) the GU site corresponding to nucleotides 264-265;
(iv) the AU site corresponding to nucleotides 271-272;
(v) the AU site corresponding to nucleotides 292-293;
(vi) the AU site corresponding to nucleotides 301-302;
(vii) the GU site corresponding to nucleotides 303-304; and
(viii) the AU site corresponding to nucleotides 316-317.

In a further preferred embodiment, the DNAzyme has a sequence selected from:

(i) 5'-caggggacaGGCTAGCTACAACGAcgttgcggg (SEQ ID NO:3)
targets GU (bp 198, 199); arms hybridise to bp 189-207

(ii) 5'-tgcaggggaGGCTAGCTACAACGAaccgttgcg (SEQ ID NO:6)
targets GU (bp 200, 201); arms hybridise to bp 191-209

(iii) 5'-catcctggaGGCTAGCTACAACGAgagcaggct (SEQ ID NO:7)
targets GU (bp 264, 265); arms hybridise to bp 255-273

(iv) 5'-ccgcggccaGGCTAGCTACAACGAcctggacga (SEQ ID NO:8)
targets AU (bp 271, 272); arms hybridise to bp 262-280

(v) 5'-ccgctgccaGGCTAGCTACAACGAcccggacgt (SEQ ID NO:9)
targets AU (bp 271, 272); arms hybridise to bp 262-280

(vi) 5'-gcggggacaGGCTAGCTACAACGAcagctgcat (SEQ ID NO:10)
targets AU (bp 301, 302); arms hybridise to bp 292-310

(vii) 5'-cagcggggaGGCTAGCTACAACGAatcagctgc (SEQ ID NO:11)
targets GU (bp 303, 304); arms hybridise to bp 294-312

(viii) 5'-ggtcagagaGGCTAGCTACAACGActgcagcgg (SEQ ID NO:12)
targets AU (bp 316, 317); arms hybridise to bp 307-325.

In a particularly preferred embodiment, the DNAzyme targets the the GU site corresponding to nucleotides 198-199, the AU site corresponding to nucleotides 271-272 or the AU site corresponding to nucleotides 301-302.

In a further preferred embodiment, the DNAzyme has the sequence: 5'-caggggacaGGCTAGCTACAACGAcgttgcggg (SEQ ID NO:3), 5'-gcggggacaGGCTAGCTACAACGAcagctgcat (SEQ ID NO:10), 5'-ccgcggccaGGCTAGCTACAACGAcctggacga (SEQ ID NO:8) or 5'-ccgctgccaGGCTAGCTACAACGAcccggacgt (SEQ ID NO:9).

In applying DNAzyme-based treatments, it is preferable that the DNAzymes be as stable as possible against degradation in the intra-cellular milieu. One means of accomplishing this is by incorporating a 3'-3' inversion at one or more termini of the DNAzyme. More specifically, a 3'-3' inversion (also referred to herein simply as an "inversion") means the covalent phosphate bonding between the 3' carbons of the terminal nucleotide and its adjacent nucleotide. This type of bonding is opposed to the normal phosphate bonding between the 3' and 5' carbons of adjacent nucleotides, hence the term "inversion". Accordingly, in a preferred embodiment, the 3'-end nucleotide residue is inverted in the building domain contiguous with the 3' end of the catalytic domain. In addition to inversions, the instant DNAzymes may contain modified nucleotides. Modified nucleotides include, for example, N3'-P5' phosphoramidate linkages, and peptide-nucleic acid linkages. These are well known in the art.

In a particularly preferred embodiment, the DNAzyme includes an inverted T at the 3' position.

Although the subject may be any animal or human, it is preferred that the subject is a human.

Within the context of the present invention, the EGR inhibitory agents may be administered either alone or in combination with one or more additional anti-cancer agents which will be known to a person skilled in the art.

Administration of the inhibitory agents may be effected or performed using any of the various methods and delivery systems known to those skilled in the art. The administering can be performed, for example, intravenously, orally, via implant, transmucosally, transdermally, topically, intramuscularly, subcutaneously or extracorporeally. In addition, the instant pharmaceutical compositions ideally contain one or more routinely used

pharmaceutically acceptable carriers. Such carriers are well known to those skilled in the art. The following delivery systems, which employ a number of routinely used carriers, are only representative of the many embodiments envisioned for administering the instant composition. In one embodiment the delivery vehicle contains Mg^{2+} or other cation(s) to serve as co-factor(s) for efficient DNAzyme bioactivity.

Transdermal delivery systems include patches, gels, tapes and creams, and can contain excipients such as solubilizers, permeation enhancers (e.g., fatty acids, fatty acid esters, fatty alcohols and amino acids), hydrophilic polymers (e.g., polycarbophil and polyvinylpyrolidone), and adhesives and tackifiers (e.g., polyisobutylenes, silicone-based adhesives, acrylates and polybutene).

Transmucosal delivery systems include patches, tablets, suppositories, pessaries, gels and creams, and can contain excipients such as solubilizers and enhancers (e.g., propylene glycol, bile salts and amino acids), and other vehicles (e.g., polyethylene glycol, fatty acid esters and derivatives, and hydrophilic polymers such as hydroxypropylmethylcellulose and hyaluronic acid).

Oral delivery systems include tablets and capsules. These can contain excipients such as binders (e.g., hydroxypropylmethylcellulose, polyvinyl pyrilodone, other cellulosic materials and starch), diluents (e.g., lactose and other sugars, starch, dicalcium phosphate and cellulosic materials), disintegrating agents (e.g., starch polymers and cellulosic materials) and lubricating agents (e.g., stearates and talc).

Solutions, suspensions and powders for reconstitutable delivery systems include vehicles such as suspending agents (e.g., gums, zanthans, cellulosics and sugars), humectants (e.g., sorbitol), solubilizers (e.g., ethanol, water, PEG and propylene glycol), surfactants (e.g., sodium lauryl sulfate, Spans, Tweens, and cetyl pyridine), preservatives and antioxidants (e.g., parabens, vitamins E and C, and ascorbic acid), anti-caking agents, coating agents, and chelating agents (e.g., EDTA).

Topical delivery systems include, for example, gels and solutions, and can contain excipients such as solubilizers, permeation enhancers (e.g., fatty acids, fatty acid esters, fatty alcohols and amino acids), and hydrophilic polymers (e.g., polycarbophil and polyvinylpyrolidone). In the preferred embodiment, the pharmaceutically acceptable carrier is a liposome or a

biodegradable polymer. Examples of carriers which can be used in this invention include the following: (1) Fugene6® (Roche); (2) SUPERFECT®(Qiagen); (3) Lipofectamine 2000®(GIBCO BRL); (4) CellFectin, 1:1.5 (M/M) liposome formulation of the cationic lipid N,NI,NII,NIII-tetramethyl-N,NI,NII,NIII-tetrapalmitylspermine and dioleoyl phosphatidyl-ethanolamine (DOPE)(GIBCO BRL); (5) Cytofectin GSV, 2:1 (M/M) liposome formulation of a cationic lipid and DOPE (Glen Research); (6) DOTAP (N-[1-(2,3-dioleoyloxy)-N,N,N-trimethyl-ammoniummethylsulfate) (Boehringer Manheim); and (7) Lipofectamine, 3:1 (M/M) liposome formulation of the polycationic lipid DOSPA and the neutral lipid DOPE (GIBCO BRL).

In a preferred embodiment, the agent is injected into or proximal the solid tumour. Injectable drug delivery systems include solutions, suspensions, gels, microspheres and polymeric injectables, and can comprise excipients such as solubility-altering agents (e.g., ethanol, propylene glycol and sucrose) and polymers (e.g., polycaprylactones and PLGA's). Implantable systems include rods and discs, and can contain excipients such as PLGA and polycaprylactone.

Delivery of the nucleic acid agents described may also be achieved via one or more, of the following non-limiting examples of vehicles:

(a) liposomes and liposome-protein conjugates and mixtures;

(b) non-liposomal lipid and cationic lipid formulations;

(c) activated dendrimer formulations;

(d) within polymer formulations such pluronic gels or within ethylene vinyl acetate coploymer (EVAc). The polymer may be delivered intra-luminally;

(e) within a viral-liposome complex, such as Sendai virus; or

(f) as a peptide-DNA conjugate.

Determining the prophylactically effective dose of the instant pharmaceutical composition can be done based on animal data using routine computational methods. In one embodiment, the prophylactically effective does contains between about 0.1 mg and about 1 g of the instant DNAzyme. In another embodiment, the prophylactically effective dose contains between about 1 mg and about 100 mg of the instant DNAzyme. In a further embodiment, the prophylactically effective does contains between about 10 mg and about 50 mg of the instant DNAzyme. In yet a further embodiment,

the prophylactically effective does contains about 25 mg of the instant DNAzyme.

It is also envisaged that nucleic acid agents targeting EGR may be administered by *ex vivo* transfection of cell suspensions, thereby inhibiting tumour growth, differentiation and/or metastasis.

In a second aspect, the present invention provides a method for inhibiting the growth or proliferation of a tumour cell, the method comprising contacting a tumour cell with an agent which inhibits induction of EGR, an agent which decreases expression of EGR or an agent which decreases the nuclear accumulation or activity of EGR.

In a third aspect, the present invention provides a tumour cell which has been transformed by introducing into the cell a nucleic acid molecule, the nucleic acid molecule comprising or encoding (i) an agent which inhibits induction of EGR, (ii) an agent which decreases expression of EGR, or (iii) an agent which decreases the nuclear accumulation or activity of EGR.

In a preferred embodiment of the third and fourth aspects, the agent is selected from the group consisting of an EGR antisense oligonucleotide or mRNA, a sequence-specific ribozyme targeted against EGR, a ssDNA targeted against EGR dsDNA and a sequence specific DNAzyme targeted against EGR.

In a fourth aspect, the present invention provides a method of screening for an agent which inhibits angiogenesis, the method comprising testing a putative agent for the ability to inhibit induction of EGR, decrease expression of EGR or decrease the nuclear accumulation or activity of EGR.

The putative agent may be tested for the ability to inhibit EGR by any suitable means. For example, the test may involve contacting a cell which expresses EGR with the putative agent and monitoring the production of EGR mRNA (by, for example, Northern blot analysis) or EGR protein (by, for example, immunohistochemical analysis or Western blot analysis). Other suitable tests will be known to those skilled in the art.

For reference, Table 1 below sets forth a comparison between the DNA sequences of mouse, rat and human EGR-1.

Table 1
Mouse, Rat and Human EGR-1

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Symbol comparison table: GenRunData:pileupdna.cmp  CompCheck: 6876
                    GapWeight: 5.000
                GapLengthWeight: 0.300
 EGR1align.msf  MSF: 4388  Type: N  April  7, 1998 12:07  Check: 5107
Name: mouseEGR1    Len:  4388  Check: 8340  Weight:  1.00 (SEQ ID NO:13)
Name: ratEGR1      Len:  4388  Check: 8587  Weight:  1.00 (SEQ ID NO:14)
Name: humanEGR1    Len:  4388  Check: 8180  Weight:  1.00 (SEQ ID NO:15)

NB. THIS IS RAT NGFI-A numbering
                1                                                    50
mouseEgr1       .......... .......... .......... .......... ..........
  ratNGFIA CCGCGGAGCC TCAGCTCTAC GCGCCTGGCG CCCTCCCTAC GCGGGCGTCC
humanEGR1       .......... .......... .......... .......... ..........

               51                                                   100
mouseEGR1       .......... .......... .......... .......... ..........
  ratEGR1   CCGACTCCCG CGCGCGTTCA GGCTCCGGGT TGGGAACCAA GGAGGGGGAG
humanEGR1       .......... .......... .......... .......... ..........

              101                                                   150
mouseEGR1       .......... .......... .......... .......... ..........
  ratEGR1   GGTGGGTGCG CCGACCCGGA AACACCATAT AAGGAGCAGG AAGGATCCCC
humanEGR1       .......... .......... .......... .......... ..........

              151                                                   200
mouseEGR1       .......... .......... .......... .......... ..........
  ratEGR1   CGCCGGAACA GACCTTATTT GGGCAGCGCC TTATATGGAG TGGCCCAATA
humanEGR1       .......... .......... .......... .......... ..........

              201                                                   250
mouseEGR1       .......... .......... .......... .......... ..........
  ratEGR1   TGGCCCTGCC GCTTCCGGCT CTGGGAGGAG GGGCGAACGG GGGTTGGGGC
humanEGR1       .......... .......... .......... .......... ..........

              251                                                   300
mouseEGR1       .......... .......... .......... .......... ..........
  ratEGR1   GGGGGCAAGC TGGGAACTCC AGGAGCCTAG CCCGGGAGGC CACTGCCGCT
humanEGR1       .......... .......... .......... .......... ..........

              301                                                   350
mouseEGR1       .......... .......... .......... .......... ..........
  ratEGR1   GTTCCAATAC TAGGCTTTCC AGGAGCCTGA GCGCTCAGGG TGCCGGAGCC
humanEGR1       .......... .......... .......... .......... ..........

              351                                                   400
mouseEGR1       .......... .......... .......... .......... ..........
  ratEGR1   GGTCGCAGGG TGGAAGCGCC CACCGCTCTT GGATGGGAGG TCTTCACGTC
humanEGR1       .......... .......... .......... .......... ..........

              401                                                   450
mouseEGR1       .......... .......... .......... .......... ..........
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      ratEGR1  ACTCCGGGTC CTCCCGGTCG GTCCTTCCAT ATTAGGGCTT CCTGCTTCCC
    humanEGR1  .......... .......... .......... .......... ..........

               451                                                 500
 5  mouseEGR1  .......... .......... .......... .......... ..........
      ratEGR1  ATATATGGCC ATGTACGTCA CGGCGGAGGC GGGCCCGTGC TGTTTCAGAC
    humanEGR1  .......... .......... .......... .......... ..........

               501                                                 550
10  mouseEGR1  .......... .......... .......... .......... ..........
      ratEGR1  CCTTGAAATA GAGGCCGATT CGGGGAGTCG CGAGAGATCC CAGCGCGCAG
    humanEGR1  .......... .......... .......... .......... ....CCGCAG

               551                                                 600
15  mouseEGR1  .....GGGGA GCCGCCGCCG CGATTCGCCG CCGCCGCCAG CTTCCGCCGC
      ratEGR1  AACTTGGGGA GCCGCCGCCG CGATTCGCCG CCGCCGCCAG CTTCCGCCGC
    humanEGR1  AACTTGGGGA GCCGCCGCCG CCATCCGCCG CCGCAGCCAG CTTCCGCCGC

               601                                                 650
20  mouseEGR1  CGCAAGATCG GCCCCTGCCC CAGCCTCCGC GGCAGCCCTG CGTCCACCAC
      ratEGR1  CGCAAGATCG GCCCCTGCCC CAGCCTCCGC GGCAGCCCTG CGTCCACCAC
    humanEGR1  CGCAGGACCG GCCCCTGCCC CAGCCTCCGC AGCCGCGGCG CGTCCACGCC

               651                                                 700
25  mouseEGR1  GGGCCGCGGC TACCGCCAGC CTGGGGGCCC ACCTACACTC CCCGCAGTGT
      ratEGR1  GGGCCGCGGC CACCGCCAGC CTGGGGGCCC ACCTACACTC CCCGCAGTGT
    humanEGR1  CGCCCGCGCC CAGGGCGAGT CGGGGTCGCC GCCTGCACGC TTCTCAGTGT

               701                                                 750
30  mouseEGR1  GCCCCTGCAC CCCGCATGTA ACCCGGCCAA CCCCCGGCGA GTGTGCCCTC
      ratEGR1  GCCCCTGCAC CCCGCATGTA ACCCGGCCAA CATCCGGCGA GTGTGCCCTC
    humanEGR1  TCCCC.GCGC CCCGCATGTA ACCCGGCCAG GCCCCCGCAA CGGTGTCCCC

               751                                                 800
35  mouseEGR1  AGTAGCTTCG GCCCCGGGCT GCGCCCACC. .ACCCAACAT CAGTTCTCCA
      ratEGR1  AGTAGCTTCG GCCCCGGGCT GCGCCCACC. .ACCCAACAT CAGCTCTCCA
    humanEGR1  TGCAGCTCCA GCCCCGGGCT GCACCCCCCC GCCCCGACAC CAGCTCTCCA

               801                                                 850
40  mouseEGR1  GCTCGCTGGT CCGGGATGGC AGCGGCCAAG GCCGAGATGC AATTGATGTC
      ratEGR1  GCTCGCACGT CCGGGATGGC AGCGGCCAAG GCCGAGATGC AATTGATGTC
    humanEGR1  GCCTGCTCGT CCAGGATGGC CGCGGCCAAG GCCGAGATGC AGCTGATGTC

               ED5 (rat) arms hybridise to bp 807-825 in rat sequ
45             hED5(hum) arms hybridise to bp 262-280 in hum sequ

               851                                                 900
    mouseEGR1  TCCGCTGCAG ATCTCTGACC CGTTCGGCTC CTTTCCTCAC TCACCCACCA
      ratEGR1  TCCGCTGCAG ATCTCTGACC CGTTCGGCTC CTTTCCTCAC TCACCCACCA
50  humanEGR1  CCCGCTGCAG ATCTCTGACC CGTTCGGATC CTTTCCTCAC TCGCCCACCA

               901                                                 950
    mouseEGR1  TGGACAACTA CCCCAAACTG GAGGAGATGA TGCTGCTGAG CAACGGGGCT
      ratEGR1  TGGACAACTA CCCCAAACTG GAGGAGATGA TGCTGCTGAG CAACGGGGCT
55  humanEGR1  TGGACAACTA CCCTAAGCTG GAGGAGATGA TGCTGCTGAG CAACGGGGCT

               951                                                1000
    mouseEGR1  CCCCAGTTCC TCGGTGCTGC CGGAACCCCA GAGGGCAGCG GCGGTAAT..
      ratEGR1  CCCCAGTTCC TCGGTGCTGC CGGAACCCCA GAGGGCAGCG GCGGCAATAA
60  humanEGR1  CCCCAGTTCC TCGGCGCCGC CGGGGCCCCA GAGGGCAGCG GCAGCAACAG
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                1001                                                1050
    mouseEGR1   .......AGC AGCAGCAGCA CCAGCAGCGG GGGCGGTGGT GGGGGCGGCA
      ratEGR1   CAGCAGCAGC AGCAGCAGCA GCAGCAGCGG GGGCGGTGGT GGGGGCGGCA
humanEGR1   CAGCAGCAGC AGCAGCGGGG GCGGTGGAGG CGGCGGGGGC GGCAGCAACA

                1051                                                1100
    mouseEGR1   GCAACAGCGG CAGCAGCGCC TTCAATCCTC AAGGGGAGCC GAGCGAACAA
      ratEGR1   GCAACAGCGG CAGCAGCGCT TTCAATCCTC AAGGGGAGCC GAGCGAACAA
humanEGR1   GCAGCAGCAG CAGCAGCACC TTCAACCCTC AGGCGGACAC GGGCGAGCAG

                1101                                                1150
    mouseEGR1   CCCTATGAGC ACCTGACCAC AG...AGTCC TTTTCTGACA TCGCTCTGAA
      ratEGR1   CCCTACGAGC ACCTGACCAC AGGTAAGCGG TGGTCTGCGC CGAGGCTGAA
humanEGR1   CCCTACGAGC ACCTGACCGC AG...AGTCT TTTCCTGACA TCTCTCTGAA

                1151                                                1200
    mouseEGR1   TAATGAGAAG GCGATGGTGG AGACGAGTTA TCCCAGCCAA ACGACTCGGT
      ratEGR1   TCCCCCTTCG TGACTACCCT AACGTCCAGT CCTTTGCAGC ACGGACCTGC
humanEGR1   CAACGAGAAG GTGCTGGTGG AGACCAGTTA CCCCAGCCAA ACCACTCGAC

                1201                                                1250
    mouseEGR1   TGCCTCCCAT CACCTATACT GGCCGCTTCT CCCTGGAGCC CGCACCCAAC
      ratEGR1   ATCTAGATCT TAGGGACGGG ATTGGGATTT CCCTCTATTC ..CACACAGC
humanEGR1   TGCCCCCCAT CACCTATACT GGCCGCTTTT CCCTGGAGCC TGCACCCAAC

                1251                                                1300
    mouseEGR1   AGTGGCAACA CTTTGTGGCC TGAACCCCTT TTCAGCCTAG TCAGTGGCCT
      ratEGR1   TCCAGGGACT TGTGTTAGAG GGATGTCTGG GGACCCCCCA ACCCTCCATC
humanEGR1   AGTGGCAACA CCTTGTGGCC CGAGCCCCTC TTCAGCTTGG TCAGTGGCCT

                1301                                                1350
    mouseEGR1   CGTGAGCATG ACCAATCCTC CGACCTCTTC ATCCTCGGCG CCTTCTCCAG
      ratEGR1   CTTGCGGGTG CGCGGAGGGC AGACCGTTTG TTTTGGATGG AGAACTCAAG
humanEGR1   AGTGAGCATG ACCAACCCAC CGGCCTCCTC GTCCTCAGCA CCATCTCCAG

                1351                                                1400
    mouseEGR1   CTGCTTCATC GTCTTCCTCT GCCTCCCAGA GCCCGCCCCT GAGCTGTGCC
      ratEGR1   TTGCGTGGGT GGCT...... .....GGAGT GGGGGAGGGT TTGTTTTGAT
humanEGR1   CGGCCTCCTC CGC...CTCC GCCTCCCAGA GCCCACCCCT GAGCTGCGCA

                1401                                                1450
    mouseEGR1   GTGCCGTCCA ACGACAGCAG TCCCATCTAC TCGGCTGCGC CCACCTTTCC
      ratEGR1   GAGCAGGGTT GC....CCCC TCCCCCGCGC GCGTTGTCGC GAGCCTTGTT
humanEGR1   GTGCCATCCA ACGACAGCAG TCCCATTTAC TCAGCGGCAC CCACCTTCCC

                1451                                                1500
    mouseEGR1   TACTCCCAAC ACTGACATTT TTCCTGAGCC CCAAAGCCAG GCCTTTCCTG
      ratEGR1   TGCAGCTTGT TCCCAAGGAA GGGCTGAAAT CTGTCACCAG GGATGTCCCG
humanEGR1   CACGCCGAAC ACTGACATTT TCCCTGAGCC ACAAAGCCAG GCCTTCCCGG

                1501                                                1550
    mouseEGR1   GCTCGGCAGG CACAGCCTTG CAGTACCCGC CTCCTGCCTA CCCTGCCACC
      ratEGR1   CCGCCCAGGG TAGGGGCGCG CATTAGCTGT GGCC.ACTAG GGTGCTGGCG
humanEGR1   GCTCGGCAGG GACAGCGCTC CAGTACCCGC CTCCTGCCTA CCCTGCCGCC

                1551                                                1600
    mouseEGR1   AAAGGTGGTT TCCAGGTTCC CATGATCCCT GACTATCTGT TTCCACAACA
      ratEGR1   GGATTCCCTC ACCCCGGACG CCTGCTGCGG AGCGCTCTCA GAGCTGCAGT
humanEGR1   AAGGGTGGCT TCCAGGTTCC CATGATCCCC GACTACCTGT TTCCACAGCA
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                1601                                                1650
    mouseEGR1   ACAGGGAGAC CTGAGCCTGG GCACCCCAGA CCAGAAGCCC TTCCAGGGTC
      ratEGR1   AGAGGGGGAT TCTCTGTTTG CGTCAGCTGT CGAAATGGCT CT......GC
5   humanEGR1   GCAGGGGGAT CTGGGCCTGG GCACCCCAGA CCAGAAGCCC TTCCAGGGCC

                1651                                                1700
    mouseEGR1   TGGAGAACCG TACCCAGCAG CCTTCGCTCA CTCCACTATC CACTATTAAA
      ratEGR1   CACTGGAGCA GGTCCAGGAA CATTGCAATC TGCTGCTATC AATTATTAAC
10  humanEGR1   TGGAGAGCCG CACCCAGCAG CCTTCGCTAA CCCCTCTGTC TACTATTAAG

                1701                                                1750
    mouseEGR1   GCCTTCGCCA CTCAGTCGGG CTCCCAGGAC TTAAAG.... ...GCTCTTA
      ratEGR1   CACATCGAGA GTCAGTGGTA GCCGGGCGAC CTCTTGCCTG GCCGCTTCGG
15  humanEGR1   GCCTTTGCCA CTCAGTCGGG CTCCCAGGAC CTGAAG.... ...GCCCTCA

                1751                                                1800
    mouseEGR1   ATACCACCTA CCAATCCCAG CTCATCA..A ACCCAGCCGC ATGCGCAAGT
      ratEGR1   CTCTCATCGT CCAGTGATTG CTCTCCAGTA ACCAGGCCTC TCTGTTCTCT
20  humanEGR1   ATACCAGCTA CCAGTCCCAG CTCATCA..A ACCCAGCCGC ATGCGCAAGT

                1801                                                1850
    mouseEGR1   ACCCCAACCG GCCCAGCAAG ACACCCCCCC ATGAACGCCC ATATGCTTGC
      ratEGR1   TTCCTGCCAG AGTCCTTTTC TGACATCGCT CTGAATAACG AGAAG..GCG
25  humanEGR1   ATCCCAACCG GCCCAGCAAG ACGCCCCCCC ACGAACGCCC TTACGCTTGC

                1851                                                1900
    mouseEGR1   CCTGTCGAGT CCTGCGATCG CCGCTTTTCT CGCTCGGATG AGCTTACCCG
      ratEGR1   CTGGTGGAGA CAAGTTATCC CAGCCAAACT ACCCGGTTGC CTCCCATCAC
30  humanEGR1   CCAGTGGAGT CCTGTGATCG CCGCTTCTCC CGCTCCGACG AGCTCACCCG

                1901                                                1950
    mouseEGR1   CCATATCCGC ATCCACACAG GCCAGAAGCC CTTCCAGTGT CGAATCTGCA
      ratEGR1   CTATACTGGC CGCTTCTCCC TGGAGCCTGC ACCCAACAGT GGCAACACTT
35  humanEGR1   CCACATCCGC ATCCACACAG GCCAGAAGCC CTTCCAGTGC CGCATCTGCA

                1951                                                2000
    mouseEGR1   TGCGTAACTT CAGTCGTAGT GACCACCTTA CCACCCACAT CCGCACCCAC
      ratEGR1   TGTGGCCTGA ACCCCTTTTC AGCCTAGTCA GTGGCCTTGT GAGCATGACC
40  humanEGR1   TGCGCAACTT CAGCCGCAGC GACCACCTCA CCACCCACAT CCGCACCCAC

                2001                                                2050
    mouseEGR1   ACAGGCGAGA AGCCTTTTGC CTGTGACATT TGTGGGAGGA AGTTTGCCAG
      ratEGR1   AACCCTCCAA CCTCTTCATC CTCAGCGCCT TCTCCAGCTG CTTCATCGTC
45  humanEGR1   ACAGGCGAAA AGCCCTTCGC CTGCGACATC TGTGGAAGAA AGTTTGCCAG

                2051                                                2100
    mouseEGR1   GAGTGATGAA CGCAAGAGGC ATACCAAAAT CCATTTAAGA CAGAAGGACA
      ratEGR1   TTCCTCTGCC TCCCAGAGCC CACCCCTGAG CTGTGCCGTG CCGTCCAACG
50  humanEGR1   GAGCGATGAA CGCAAGAGGC ATACCAAGAT CCACTTGCGG CAGAAGGACA

                2101                                                2150
    mouseEGR1   AGAAAGCAGA CAAAAGTGTG GTGGCCTCCC CGGCTGC... .CTCTTCACT
      ratEGR1   ACAGCAGTCC CATTTACTCA GCTGCACCCA CCTTTCCTAC TCCCAACACT
55  humanEGR1   AGAAAGCAGA CAAAAGTGTT GTGGCCTCTT CGGCCACCTC CTCTCTCTCT

                2151                                                2200
    mouseEGR1   .......... .......... CTCTTCTTAC CCATCCCCAG TGGCTACCTC
      ratEGR1   .......... .......... GACATTTTTC CTGAGCCCCA AAGCCAGGCC
60  humanEGR1   TCCTACCCGT CCCCGGTTGC TACCTCTTAC CCGTCCCCGG TTACTACCTC
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                2201                                                2250
    mouseEGR1   CTACCCATCC CCTGCCACCA CCTCATTCCC ATCCCCTGTG CCCACTTCCT
      ratEGR1   TTTCCTGGCT CTGCAGGCAC AGCCTTGCAG TACCCGCCTC CTGCCTACCC
    humanEGR1   TTATCCATCC CCGGCCACCA CCTCATACCC ATCCCCTGTG CCCACCTCCT

                2251                                                2300
    mouseEGR1   ACTCCTCTCC TGGCTCCTCC ACCTACCCAT CTCCTGCGCA CAGTGGCTTC
      ratEGR1   TGCCACCAAG GGTGGTTTCC AGGTTCCCAT GATCCCTGAC TATCTGTTTC
    humanEGR1   TCTCCTCTCC CGGCTCCTCG ACCTACCCAT CCCCTGTGCA CAGTGGCTTC

                2301                                                2350
    mouseEGR1   CCGTCGCCGT CAGTGGCCAC CACCTTTGCC TCCGTTCC.. ..........
      ratEGR1   CACAACAACA GGGAGACCTG AGCCTGGGCA CCCCAGACCA GAAGCCCTTC
    humanEGR1   CCCTCCCCGT CGGTGGCCAC CACGTACTCC TCTGTTCCC. ..........

                2351                                                2400
    mouseEGR1   ....ACCTGC TTTCCCCACC CAGGTCAGCA GCTTCCCGTC TGCGGGCGTC
      ratEGR1   CAGGGTCTGG AGAACCGTAC CCAGCAGCCT TCGCTCACTC CACTATCCAC
    humanEGR1   .....CCTGC TTTCCCGGCC CAGGTCAGCA GCTTCCCTTC CTCAGCTGTC

                2401                                                2450
    mouseEGR1   AGCAGCTCCT TCAGCACCTC AACTGGTCTT TCAGACATGA CAGCGACCTT
      ratEGR1   TATCAAAGCC TTCGCCACTC AGTCGGGCTC CCAGGACTTA AAGGCTCTTA
    humanEGR1   ACCAACTCCT TCAGCGCCTC CACAGGGCTT TCGGACATGA CAGCAACCTT

                2451                                                2500
    mouseEGR1   TTCTCCCAGG ACAATTGAAA TTTGCTAAAG GGA....... .ATAAAAG..
      ratEGR1   ATAACACCTA CCAGTCCCAA CTCATCAAAC CCAGCCGCAT GCGCAAGT..
    humanEGR1   TTCTCCCAGG ACAATTGAAA TTTGCTAAAG GGAAAGGGGA AAGAAAGGGA

                2501                                                2550
    mouseEGR1   .AAAGCAAAG GGAGAGGCAG GAAAGACATA AAAGCA...C AGGAGGGAAG
      ratEGR1   .ACCCCAACC GGCCCAGCAA GACACCCCCC CATGAACGCC CGTATGCTTG
    humanEGR1   AAAGGGAGAA AAAGAAACAC AAGAGACTTA AAGGACAGGA GGAGGAGATG

                2551                                                2600
    mouseEGR1   AGATGGCCGC AAGAGGGGCC ACCTCTTAGG TCAGATGGAA GATCTCAGAG
      ratEGR1   CCCTGTTGAG TCCTGCGATC GCCGCTTTTC TCGCTCGGAT GAGCTTACAC
    humanEGR1   GCCATAGGAG AGGAGGGTT. .CCTCTTAGG TCAGATGGAG GTTCTCAGAG

                2601                                                2650
    mouseEGR1   CCAAGTCCTT CTACTCACGA GTA..GAAGG ACCGTTGGCC AACAGCCCTT
      ratEGR1   GCCACATCCG CATCCATACA GGC..CAGAA GCCCTTCCAG TGTCGAATCT
    humanEGR1   CCAAGTCCTC CCTCTCTACT GGAGTGGAAG GTCTATTGGC CAACAATCCT

                2651                                                2700
    mouseEGR1   TCACTTACCA TCCCTGCCTC CCCCGTCCTG TTCCCTTTGA CTTCAGCTGC
      ratEGR1   GCATGCGTAA TTTCAGTCGT AGTGACCACC TTACCACCCA CATCCGCACC
    humanEGR1   TTCTGCCCAC TTCCCCTTCC CCAATTACTA TTCCCTTTGA CTTCAGCTGC

                2701                                                2750
    mouseEGR1   CTGAAACAGC CATGTCCAAG TTCTTCACCT CTATCCAAAG GACTTGATTT
      ratEGR1   C..ACACAGG CGAGAAGCCT TTTGCCTGTG ACATTTGTGG GAGAAAGTTT
    humanEGR1   CTGAAACAGC CATGTCCAAG TTCTTCACCT CTATCCAAAG AACTTGATTT

                2751                                                2800
    mouseEGR1   GCATGG.... ..TATTGGAT AAATCATTTC AGTATCCTCT ..........
      ratEGR1   GCCAGGAGTG ATGAACGCAA GAGGCATACC AAAATCCACT TAAGACAGAA
    humanEGR1   GCATGGA... ..TTTTGGAT AAATCATTTC AGTATCATCT ..........
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                2801                                                2850
     mouseEGR1  .....CCATC ACATGCCTGG CCCTTGCTCC CTTCAGCGCT AGACCATCAA
       ratEGR1  GGACAAGAAA GCAGACAAAA GTGTCGTGGC CTCCTCAGCT GCCTCTTCCC
5    humanEGR1  ....CCATCA TATGCCTGAC CCCTTGCTCC CTTCAATGCT AGAAAATCGA

                2851                                                2900
     mouseEGR1  GTTGGCATAA AGAAAAAAAA ATGGGTTTGG GCCCTCAGAA CCCTGCCCTG
       ratEGR1  TCTCTTCCTA CCCATCCCCA GTGGCTACCT CCTACCCATC CCCCGCCACC
10   humanEGR1  GTTGGC.... .....AAAAT GGGGTTTGGG CCCCTCAGAG CCCTGCCCTG

                2901                                                2950
     mouseEGR1  CATCTTTGTA CAGCATCTGT GCCATGGATT TTGTTTTCCT TGGGGTATTC
       ratEGR1  ACCTCATTTC CATCCCCAGT GCCCACCTCT TACTCCTCTC CGGGCTCCTC
15   humanEGR1  CACCCTTGTA CAGTGTCTGT GCCATGGATT TCGTTTTTCT TGGGGTACTC

                2951                                                3000
     mouseEGR1  TTGATGTGAA GATAATTTGC ATACT..... .CTATTGTAT TATTTGGAGT
       ratEGR1  TACCTACCCG TCTCCTGCAC ACAGTGGCTT CCCATCGCCC TCGGTGGCCA
20   humanEGR1  TTGATGTGAA GATAATTTGC ATATT..... .CTATTGTAT TATTTGGAGT

                3001                                                3050
     mouseEGR1  TAAATCCTCA CTTTGGGG.. GAGGGGGGAG CAAAGCCAAG CAAACCAATG
       ratEGR1  CCACCTATGC CTCCGTCC.. CACCTGCTTT CCCTGCCCAG GTCAGCACCT
25   humanEGR1  TAGGTCCTCA CTTGGGGGAA AAAAAAAAAA AAAAGCCAAG CAAACCAATG

                3051                                                3100
     mouseEGR1  ATGATCCTCT ATTTTGTGAT GACTCTGCTG TGACATTA.. ..........
       ratEGR1  TCCAGTCTGC AGGGGTCAGC AACTCCTTCA GCACCTCAAC GGGTCTTTCA
30   humanEGR1  GTGATCCTCT ATTTTGTGAT GATGCTGTGA CAATA..... ..........

                3101                                                3150
     mouseEGR1  .GGTTTGAAG CATTTTTTTT TTCAAGCAGC AGTCCTAGGT ATTAACTGGA
       ratEGR1  GACATGACAG CAACCTTTTC TCCTAGGACA ATTGAAATTT GCTAAAGGGA
35   humanEGR1  ...AGTTTGA ACCTTTTTTT TTGAAACAGC AGTCCCAG.. ..TATTCTCA

                3151                                                3200
     mouseEGR1  ..GCATGTGT CAGAGTGTTG TTCCGTTAAT TTTGTAAATA CTGGCTCGAC
       ratEGR1  ATGAAAGAGA GCAAAGGGAG GGGAGCGCGA GAGACAATAA AGGACAGGAG
40   humanEGR1  GAGCATGTGT CAGAGTGTTG TTCCGTTAAC CTTTTTGTAA ATACTGCTTG

                3201                                                3250
     mouseEGR1  .TGTAACTCT CACATGTGAC AAAGTATGGT TTGTTTGGTT GGGTTTTGTT
       ratEGR1  .GGAAGAAAT GGCCCGCAAG AGGGGCTGCC TCTTAGGTCA GATGGAAGAT
45   humanEGR1  ACCGTACTCT CACATGTGGC AAAATATGGT TTGGTTTTTC TTTTTTTTTT

                3251                                                3300
     mouseEGR1  TTTGAGAATT TTTTTGCCCG TCCCTTTGGT TTCAAAAGTT TCACGTCTTG
       ratEGR1  CTCAGAGCCA AGTCCTTCTA GTCAGTAGAA GGCCCGTTGG CCACCAGCCC
50   humanEGR1  TTGAAAGTGT TTTTTCTTCG TCCTTTTGGT TTAAAAAGTT TCACGTCTTG

                3301                                                3350
     mouseEGR1  GTGCCTTTTG TGTGACACGC CTT.CCGATG GCTTGACATG CGCA......
       ratEGR1  TTTCACTTAG CGTCCCTGCC CTC.CCCAGT CCCGGTCCTT TTGACTTCAG
55   humanEGR1  GTGCCTTTTG TGTGATGCCC CTTGCTGATG GCTTGACATG TGCAAT....

                3351                                                3400
     mouseEGR1  ...GATGTGA GGGACACGCT CACCTTAGCC TTAA...GGG GGTAGGAGTG
       ratEGR1  CTGCCTGAAA CAGCCACGTC CAAGTTCTTC ACCT...CTA TCCAAAGGAC
60   humanEGR1  .....TGTGA GGGACATGCT CACCTCTAGC CTTAAGGGGG GCAGGGAGTG
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                3401                                                 3450
    mouseEGR1   ATGTGTTGGG GGAGGCTTGA GAGCAAAAAC GAGGAAGAGG GCTGAGCTGA
      ratEGR1   TTGATTTGCA TGGTATTGGA TAAACCATTT CAGCATCATC TCCACCACAT
    humanEGR1   ATGATTTGGG GGAGGCTTTG GGAGCAAAAT AAGGAAGAGG GCTGAGCTGA

                3451                                                 3500
    mouseEGR1   GCTTTCGGTC TCCAGAATGT AAGAAGAAAA AATTTAAACA AAAATCTGAA
      ratEGR1   GCCTGGCCCT TGCTCCCTTC AGCACTAGAA CATCAAGTTG GCTGAAAAAA
    humanEGR1   GCTTCGGTTC TCCAGAATGT AAGAAAACAA AATCTAAAAC AAAATCTGAA

                3501                                                 3550
    mouseEGR1   CTCTCAAAAG TCTATTTTTC TAAACTGAAA ATGTAAATTT ATACATCTAT
      ratEGR1   AAAATGGGTC TGGGCCCTCA GAACCCTGCC CTGTATCTTT GTACA.....
    humanEGR1   CTCTCAAAAG TCTATTTTTT TAA.CTGAAA ATGTAAATTT ATAAATATAT

                3551                                                 3600
    mouseEGR1   TCAGGAGTTG GAGTGTTGTG GTTACCTACT GAGTAGGCTG CAGTTTTTGT
      ratEGR1   GCATCTGTGC CATGGATTTT GTTTTCCTTG GGGTATTCTT GATGTGAAGA
    humanEGR1   TCAGGAGTTG GAATGTTGTA GTTACCTACT GAGTAGGCGG CGATTTTTGT

                3601                                                 3650
    mouseEGR1   ATGTTATGAA CATGAAGTTC ATTATTTTGT GGTTTTATTT TACTTTGTAC
      ratEGR1   TAATTTGCAT ACTCTATTGT ACTATTTGGA GTTAAATTCT CACTTTGGGG
    humanEGR1   ATGTTATGAA CATGCAGTTC ATTATTTTGT GGTTCTATTT TACTTTGTAC

                3651                                                 3700
    mouseEGR1   TTGTGTTTGC TTAAACAAAG TAACCTGTTT GGCTTATAAA CACATTGAAT
      ratEGR1   GAGGGGGAGC AAAGCCAAGC AAACCAATGG TGATCCTCTA TTTTGTGATG
    humanEGR1   TTGTGTTTGC TTAAACAAAG TGA.CTGTTT GGCTTATAAA CACATTGAAT

                3701                                                 3750
    mouseEGR1   GCGCTCTATT GCCCATGG.. ..GATATGTG GTGTGTATCC TTCAGAAAAA
      ratEGR1   ATCCTGCTGT GACATTAGGT TTGAAACTTT TTTTTTTTTT TGAAGCAGCA
    humanEGR1   GCGCTTTATT GCCCATGG.. ..GATATGTG GTGTATATCC TTCCAAAAAA

                3751                                                 3800
    mouseEGR1   TTAAAAGGAA AAAT..... ......... ......... .........
      ratEGR1   GTCCTAGGTA TTAACTGGAG CATGTGTCAG AGTGTTGTTC CGTTAATTTT
    humanEGR1   TTAAAACGAA AATAAAGTAG CTGCGATTGG G......... .........

                3801                                                 3850
    mouseEGR1   ......... ......... ......... ......... .........
      ratEGR1   GTAAATACTG CTCGACTGTA ACTCTCACAT GTGACAAAAT ACGGTTTGTT
    humanEGR1   ......... ......... ......... ......... .........

                3851                                                 3900
    mouseEGR1   ......... ......... ......... ......... .........
      ratEGR1   TGGTTGGGTT TTTTGTTGTT TTTGAAAAAA AAATTTTTTT TTTGCCCGTC
    humanEGR1   ......... ......... ......... ......... .........

                3901                                                 3950
    mouseEGR1   ......... ......... ......... ......... .........
      ratEGR1   CCTTTGGTTT CAAAAGTTTC ACGTCTTGGT GCCTTTGTGT GACACACCTT
    humanEGR1   ......... ......... ......... ......... .........

                3951                                                 4000
    mouseEGR1   ......... ......... ......... ......... .........
      ratEGR1   GCCGATGGCT GGACATGTGC AATCGTGAGG GGACACGCTC ACCTCTAGCC
    humanEGR1   ......... ......... ......... ......... .........
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                4001                                                4050
    mouseEGR1   .......... .......... .......... .......... ..........
      ratEGR1   TTAAGGGGGT AGGAGTGATG TTTCAGGGGA GGCTTTAGAG CACGATGAGG
    humanEGR1   .......... .......... .......... .......... ..........

                4051                                                4100
    mouseEGR1   .......... .......... .......... .......... ..........
      ratEGR1   AAGAGGGCTG AGCTGAGCTT TGGTTCTCCA GAATGTAAGA AGAAAAATTT
    humanEGR1   .......... .......... .......... .......... ..........

                4101                                                4150
    mouseEGR1   .......... .......... .......... .......... ..........
      ratEGR1   AAAACAAAAA TCTGAACTCT CAAAAGTCTA TTTTTTTAAC TGAAAATGTA
    humanEGR1   .......... .......... .......... .......... ..........

                4151                                                4200
    mouseEGR1   .......... .......... .......... .......... ..........
      ratEGR1   GATTTATCCA TGTTCGGGAG TTGGAATGCT GCGGTTACCT ACTGAGTAGG
    humanEGR1   .......... .......... .......... .......... ..........

                4201                                                4250
    mouseEGR1   .......... .......... .......... .......... ..........
      ratEGR1   CGGTGACTTT TGTATGCTAT GAACATGAAG TTCATTATTT TGTGGTTTTA
    humanEGR1   .......... .......... .......... .......... ..........

                4251                                                4300
    mouseEGR1   .......... .......... .......... .......... ..........
      ratEGR1   TTTTACTTCG TACTTGTGTT TGCTTAAACA AAGTGACTTG TTTGGCTTAT
    humanEGR1   .......... .......... .......... .......... ..........

                4301                                                4350
    mouseEGR1   .......... .......... .......... .......... ..........
      ratEGR1   AAACACATTG AATGCGCTTT ACTGCCCATG GGATATGTGG TGTGTATCCT
    humanEGR1   .......... .......... .......... .......... ..........

                4351                                    4388
    mouseEGR1   .......... .......... .......... ........
      ratEGR1   TCAGAAAAAT TAAAAGGAAA ATAAAGAAAC TAACTGGT
    humanEGR1   .......... .......... .......... ........
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EXPERIMENTAL DETAILS

EXAMPLE 1

Role of EGR-1 in endothelial cell proliferation and migration

Materials and Methods

Oligonucleotides and chemicals. Phosphorothioate-linked antisense oligonucleotides directed against the region comprising the translational start site of Egr-1 mRNA were synthesized commercially (Genset Pacific) and purified by high performance liquid chromatography. The target sequence of AS2 (5'-CsTsTsGsGsCsCsGsCsTsGsCsCsAsT-3') (SEQ ID NO:16) is conserved in mouse, rat and human Egr-1 mRNA. For control purposes, we used AS2C (5'-GsCsAsCsTsTsCsTsGsCsTsGsTsCsC-3') (SEQ ID NO:17), a size-matched phosphorothioate-linked counterpart of AS2 with similar base composition. Phorbol-12-myristrate 13-acetate (PMA) and fibroblast growth factor-2 were purchased from Sigma-Aldrich.

Cell culture. Bovine aortic endothelial cells were obtained from Cell Applications, Inc. and used between passages 5-9. The endothelial cells were grown in Dulbecco's modified Eagles' medium (Life Technologies), pH 7.4, containing 10% fetal bovine serum supplemented with 50 μg/mL streptomycin and 50 IU/mL penicillin. The cells were routinely passaged with trypsin/EDTA and maintained at 37°C in a humidified atmosphere of 5% CO2/95% air.

Transient transfection analysis and CAT assay. The endothelial cells were grown to 60-70% confluence in 100mm dishes and transiently transfected with 10 μg of the indicated chloramphenicol acetyl transferase (CAT)-based promoter reporter construct using FuGENE6 (Roche). The cells were rendered growth-quiescent by incubation 48 h in 0.25% FBS, and stimulated with various agonists for 24 h prior to harvest and assessment of CAT activity. CAT activity was measured and normalized to the concentration of protein in the lysates (determined by Biorad Protein Assay) as previously described (Khachigian et al., 1999).

Northern blot analysis. Total RNA (12 μg/well) of growth-arrested endothelial cells (prepared using TRIzol Reagent (Life Technologies) in

accordance with the manufacturer's instructions) previously exposed to various agonists for 1 h was resolved by electrophoresis on denaturing 1% agarose-formaldehyde gels. Following transfer overnight to Hybond- N+ nylon membranes (Amersham), the blots were hybridized with ^{32}P-labeled Egr-1 cDNA prepared using the Nick Translation Kit overnight (Roche). The membranes were washed and radioactivity visualized by autoradiography as previously described (Khachigian et al., 1995).

RT-PCR. Reverse transcription was performed with 8 μg of total RNA using M-MLV reverse transcriptase. Egr-1 cDNA was amplified (334 bp product (Delbridge et al., 1997)) using Taq polymerase by heating for 1 min at 94°C, and cycling through 94°C for 1 min, 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. Following thirty cycles, a 5 min extension at 72°C was carried out. Samples were electrophoresed on 1.5% agarose gel containing ethidium bromide and photographed under ultraviolet illumination. β-actin amplification (690 bp product) was performed essentially as above. The sequences of the primers were: Egr-1 forward primer (5'-GCA CCC AAC AGT GGC AAC-3') (SEQ ID NO:18), Egr-1 reverse primer (5'-GGG ATC ATG GGA ACC TGG-3') (SEQ ID NO:19), β-actin forward primer (5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA 3') (SEQ ID NO:20), and β-actin reverse primer (5'-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG-3') (SEQ ID NO:21).

Antisense oligonucleotide delivery and Western blot analysis. Growth-arrested cells in 100 mm dishes were incubated with the indicated oligonucleotides 24 h and 48 h after the initial change of medium. When oligonucleotide was added a second time, the cells were incubated with various concentrations of insulin and harvest 1 h subsequently. The cells were washed in cold phosphate-buffered saline (PBS), pH 7.4, and solubilized in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 1% trasylol, 10 μg/ml leupeptin, 1% aprotinin, 2 μM PMSF). Lysates were resolved by electrophoresis on 8% denaturing SDS-polyacrylamide gels, transferred to PDVF nylon membranes (NEN-DuPont), blocked with skim milk powder, then incubated with polyclonal antibodies to Egr-1 (Santa Cruz Biotechnology, Inc) and monoclonal horseradish peroxidase–linked mouse anti-rabbit Ig secondary antibodies followed by chemiluminescent detection (NEN-DuPont).

^{3}H-Thymidine incorporation into DNA. Growth-arrested endothelial cells at 90% confluence in 96 well plates were incubated twice with the oligonucleotides prior to the addition of insulin. When signaling inhibitors (PD98059, SB202190, wortmannin) were used in experiments, these agents were added 2 h before the addition of insulin. After 18 h of exposure to insulin, the cells were pulsed with 200,000 cpm/well of methyl-^{3}H thymidine (NEN-DuPont) for 6 h. Lysates were prepared by washing first in cold PBS, pH7.4, then fixing with cold 10% trichloroacetic acid, washing with cold ethanol and solubilizing in 0.1 M NaOH. ^{3}H-Thymidine in the lysates was quantitated with ACSII scintillant using β-scintillation counter (Packard).

In vitro injury. Growth-arrested cells at 90% confluence were incubated with antisense oligonucleotides and insulin at various concentrations as described above, then were scraped by drawing a sterile wooden toothpick across the monolayer (Khachigian et al., 1996). Following 48-72 h, the cells were fixed in 4% formalin, stained with hematoxylin/eosin then photographed.

HMEC-1 culture and proliferation assay. SV40-transformed HMEC-1 cells were grown in MCDB 131 medium with EGF (10 ng/ml) and hydrocortisone (1 μg/ml) supplements and 10% FBS. Forty-eight h after incubation in serum-free medium without supplements, the cells were transfected with the indicated DNA enzyme (0.4 μM) and transfected again 72 h after the change of medium, when 10% serum was added. The cells were quantitated by Coulter counter, 24 h after the addition of serum.

Antisense Egr-1 mRNA overexpression. Bovine aortic endothelial cells or rat vascular smooth muscle cells were grown to 60% confluence in 96-well plates then transfected with 3μg of construct pcDNA3-A/SEgr-1 (in which a 137bp fragment of Egr-1 cDNA (732-869) was cloned in antisense orientation into the BamHI/EcoRI site of pcDNA3), or pcDNA3 alone, using Fugene6 in accordance with the manufacturer's instructions. Growth arrested cells were incubated with 5% FBS in Waymouth's medium (SMC) or DMEM (EC) and trypisinised after 3 days prior to quantitation of the cell populations by Coulter counting.

Results and Discussion

Insulin, but not Glucose, Stimulates Egr-1 Activity in Vascular Endothelial Cells. High glucose may activate normally-quiescent vascular

endothelium by stimulating mitogen-activated protein (MAP) kinase activity and the expression of immediate-early genes (Frodin et al., 1995; Kang et al., 1999). These signaling and transcriptional events may, in turn, induce the expression of other genes whose products then alter endothelial phenotype and facilitate the development of lesions. To determine the effect of glucose on Egr-1 activity in vascular endothelial cells, we performed transient transfection analysis in endothelial cells transfected with pEBS1^{3}foscat, a chloramphenical acetyltransferase (CAT)-based reporter vector driven by three high-affinity Egr-1 binding sites placed upstream of the c-fos TATA box (Gashler et al., 1993). Exposure of growth-arrested endothelial cells to various concentrations of glucose (5 to 30 mM) over 24 h did not increase Egr-1 binding activity (Figure 1). However, Egr-1 binding activity did increase in cells exposed to insulin (100 nM) (Figure 1). Reporter activity also increased upon incubation with FGF-2, a known inducer of Egr-1 transcription and binding activity in vascular endothelial cells (Santiago et al., 1999b) (Figure 1).

Insulin and FGF-2 Induce Egr-1 mRNA Expression in Vascular Endothelial Cells. The preceding findings using reporter gene analysis provided evidence for increased Egr-1 expression in endothelial cells exposed to insulin. We next used reverse transcription-polymerase chain reaction (RT-PCR) and Northern blot analysis to demonstrate directly the capacity of insulin to increase levels of Egr-1 mRNA. RT-PCR revealed that Egr-1 is weakly expressed in growth-quiescent endothelial cells (data not shown). Insulin, like FGF-2, increased Egr-1 expression within 1 h of exposure to the agonist. In contrast, levels of β-actin mRNA were unchanged. Northern blot analysis confirmed these qualitative data by demonstrating that insulin, FGF-2, and phorbol 12-myristate 13-acetate (PMA), a second potent inducer of Egr-1 expression (Khachigian et al., 1995) elevated steady-state Egr-1 mRNA levels within 1 h without increasing levels of ribosomal 28S and 18S mRNA (data not shown).

Insulin-Stimulated Egr-1 Protein Synthesis in Endothelial Cells is Inhibited by Antisense Oligonucleotides Targeting Egr-1 mRNA. To reconcile our demonstration of insulin-induced Egr-1 mRNA expression with the binding activity of the transcription factor (Figure 1), we performed Western immunoblot analysis using polyclonal antibodies directed against Egr-1 protein. Insulin (at 100 nM and 500 nM) induced Egr-1 protein synthesis in

growth-arrested endothelial cells within 1 h (data not shown). These findings, taken together, demonstrate that insulin elevates Egr-1 mRNA, protein and binding activity in vascular endothelial cells.

We recently developed phosphorothioate-based antisense oligonucleotides targeting the translational start site in Egr-1 mRNA (Santiago et al., 1999c). These oligonucleotides lack phosphorothioate G-quartet sequences that have been associated with non-specific biological activity (Stein, 1997). Western blot analysis revealed that prior incubation of growth-arrested endothelial cells with 0.8 μM antisense Egr-1 oligonucleotides (AS2) inhibited insulin-inducible Egr-1 protein synthesis, despite equal loading of protein. The lack of attenuation in insulin-inducible Egr-1 protein following exposure of the cells to an identical concentration of AS2C demonstrates the sequence-specific inhibitory effect of the antisense Egr-1 oligonucleotides.

Insulin Stimulates Endothelial Cell DNA Synthesis which is Inhibited by Antisense Oligonucleotides Targeting Egr-1 mRNA. These oligonucleotides, which attenuate the induction of Egr-1 protein, were used in ^{3}H-thymidine incorporation assays to determine the involvement of Egr-1 in insulin-inducible DNA synthesis. This assay evaluates ^{3}H-thymidine uptake into DNA precipitable with trichloroacetic acetic (TCA) (Khachigian et al., 1992). In initial experiments, growth-arrested endothelial cells exposed to insulin (100 nM) increased the extent of DNA synthesis by 100%, whereas 500 nM insulin caused a 200% increase in DNA synthesis (Figure 2A).

We next determined the effect of AS2 and AS2C on insulin-inducible endothelial DNA synthesis. In the absence of added insulin, AS2 (0.8 μM) inhibited basal endothelial DNA synthesis facilitated by low concentrations of serum (0.25% v:v) (Figure 2B). In contrast, the scrambled control (0.8 μM) or a third oligonucleotide, E3 (0.8 μM), a size-matched phosphorothioate directed toward another region of Egr-1 mRNA (Santiago et al., 1999c) had little effect on basal DNA synthesis (Figure 2B). Furthermore, unlike AS2 and E3, AS2 significantly inhibited DNA synthesis inducible by insulin (500 nM and 1000 nM) (Figure 2B). To demonstrate concentration-dependent inhibition of DNA synthesis, we incubated the endothelial cells with 0.4 μM as well as 0.8 μM of Egr-1 oligonucleotide. Since this lower concentration of AS2 inhibited 3H-thymidine incorporation less effectively (compare to AS2C) indicates dose-dependent and sequence-specific inhibition by the antisense

Egr-1 oligonucleotide (Figure 2C). These findings thus demonstrate the requirement for Egr-1 protein in endothelial cell DNA synthesis inducible by insulin.

Insulin-Stimulated DNA Synthesis in Endothelial Cells is Inhibited by PD98059 and Wortmannin, But Not by SB202190. Inducible Egr-1 transcription is governed by the activity of extracellular signal-regulated kinase (ERK) (Santiago et al., 1999b) which phosphorylates factors at serum response elements in the Egr-1 promoter (Gashler et al, 1995). Since there is little known about signaling pathways mediating insulin-inducible proliferation of vascular endothelial cells, we determined the relevance of MEK/ERK in this process using the specific MEK/ERK inhibitor, PD98059. This compound (at 10 and 30 μM) inhibited insulin-inducible DNA synthesis in a dose-dependent manner (Figure 3). Likewise, wortmannin (0.3 and 1 μM), the phosphatidylinositol 3-kinase inhibitor which also inhibits c-Jun N-terminal kinase (JNK) (Ishizuka et al, 1999; Day et al., 1999; Kumahara et al., 1999), ERK (Barry et al., 1999) and p38 kinase (Barry et al., 1999) inhibited DNA synthesis in a dose-dependent manner (Figure 3). In contrast, SB202190 (100 and 500 nM), a specific p38 kinase inhibitor failed to affect DNA synthesis (Figure 3). These findings demonstrate the critical role for MEK/ERK, and possibly JNK, in insulin-inducible endothelial cell proliferation, and the lack of p38 kinase involvement in this process.

Insulin Stimulates Endothelial Cell Regrowth After Mechanical Injury In Vitro in an Egr-1-Dependent Manner. Mechanically wounding vascular endothelial (and smooth muscle) cells in culture results in migration and proliferation at the wound edge and the eventual recoverage of the denuded area. We hypothesized that insulin would accelerate this cellular response to mechanical injury. Acutely scraping the growth-quiescent (rendered by 48 h incubation in 0.25% serum) endothelial monolayer resulted in a distinct wound edge (data not shown). Continued incubation of the cultures in medium containing low serum for a further 3 days resulted in weak regrowth in the denuded zone but aggressive regrowth in the presence of optimal amounts of serum (10%). When insulin (500 nM) was added to growth-quiescent cultures at the time of injury the population of cells in the denuded zone significantly increased, albeit as expected, less efficiently than the 10% serum control.

To investigate the involvement of Egr-1 in endothelial regrowth potentiated by insulin after injury we incubated the cultures with antisense Egr-1 oligonucleotides prior to scraping and again at the time of injury and the addition of insulin. AS2 (0.8 μM) significantly inhibited endothelial regrowth stimulated by insulin. In contrast, regrowth in the presence of AS2C (0.8 μM) was not significantly different from cultures in which oligonucleotide was omitted. Similar findings were observed when higher concentrations (1.2 μM) of AS2 and AS2C were used. Thus, endothelial regrowth after injury stimulated by insulin proceeds in an Egr-1-dependent manner. These observations are quantitated in Figure 4.

These results show that insulin-induced proliferation and regrowth after injury are processes critically dependent upon the activation of Egr-1. Northern blot, RT-PCR and Western immunoblot analysis reveal that insulin induces Egr-1 mRNA and protein expression. Antisense oligonucleotides which block insulin-induced synthesis of Egr-1 protein in a sequence-specific and dose-dependent manner, also inhibit proliferation and regrowth after mechanical injury. These findings using nucleic acids specifically targeting Egr-1 demonstrate the functional involvement of this transcription factor in endothelial growth.

Insulin signaling involves the activation of a growing number of immediate-early genes and transcription factors. These include c-fos (Mohn et al., 1990; Jhun et al, 1995; Harada et al., 1996), c-jun (Mohn et al., 1990), nuclear factor-κB (Bertrand et al., 1998), SOCS3 (Emanuelli et al., 2000) and the forkhead transcription factor FKHR (Nakae et al., 1999). Insulin also induces the expression of Egr-1 in mesangial cells (Solow et al., 1999), fibroblasts (Jhun et al., 1995), adipocytes (Alexander-Bridges et al., 1992) and Chinese hamster ovary cells (Harada et al., 1996). This study is the first to describe the induction of Egr-1 by insulin in vascular endothelial cells.

Insulin activates several subclasses within the MAP kinase superfamily, including ERK, JNK and p38 kinase (Guo et al., 1998). Our findings indicate that the specific ERK inhibitor PD98059, which binds to MEK and prevents phosphorylation by Raf, inhibits insulin-inducible endothelial cell proliferation. Egr-1 transcription is itself dependent upon the phosphorylation activity of ERK via its activation of ternary complex factors (such as Elk-1) at serum response elements (SRE) in the Egr-1 promoter. Six SREs appear in the Egr-1 promoter whereas only one is present

in the c-fos promoter (Gashler et al., 1995). PD98059 blocks insulin-inducible Elk-1 transcriptional activity at the c-fos SRE in vascular cells (Xi et al., 1997). These published findings are consistent with the present demonstration of the involvement of Egr-1 in insulin-inducible proliferation.

To provide evidence, independent of insulin, that endothelial proliferation is an Egr-1-dependent process, we incubated human microvascular endothelial cells (HMEC-1) separately with two DNA enzymes (DzA and DzF) each targeting different sites in human EGR-1 mRNA, at a final concentration of 0.4 μM. DzA and DzF both inhibited HMEC-1 replication (total cell counts) in the presence of 5% serum (Figure 5). In contrast, DzFscr, was unable to modulate proliferation at the same concentration (Figure 5). DzFscr bears the same active 15nt catalytic domain as DzF and has the same net charge but has scrambled hybridizing arms. These data obtained using a second endothelial cell type demonstrate inhibition of endothelial proliferation using sequence-specific strategies targeting human EGR-1.

Finally, we found that CMV-mediated overexpression of antisense Egr-1 mRNA inhibited proliferation of both endothelial cells and smooth muscle cells. Replication of both endothelial and smooth muscle cell pcDNA3-A/SEgr-1 transfectants was significantly lower than those transfected with the backbone vector alone, pcDNA3 (data not shown). These findings demonstrate that antisense EGR mRNA strategies can inhibit proliferation of arterial endothelial cells and at least one other vascular cell type.

Despite the availability and clinical use of a large number of chemotherapeutic agents for the clinical management of neoplasia, solid tumours remain a major cause of mortality in the Western world. Drugs currently used to treat such tumours are generally non-specific poisons that can be toxic to non-cancerous tissue and require high doses for efficacy. There is growing evidence that the cellular and molecular mechanisms underlying tumour growth involves more than just tumour cell proliferation and migration. Importantly, tumour growth and metastasis are critically dependent upon ongoing angiogenesis, the process new blood vessel formation (Crystal et al., 1999). The present findings, which demonstrate that Egr-1 is critical in vascular endothelial cell replication and migration, strongly implicate this transcription factor as a key regulator in angiogenesis and tumorigenesis.

Example 2

Characterisation of DNAzyme targeting rat Egr-1 (NGFI-A)

Materials and Methods

ODN synthesis. DNAzymes were synthesized commercially (Oligos Etc., Inc.) with an inverted T at the 3' position unless otherwise indicated. Substrates in cleavage reactions were synthesized with no such modification. Where indicated ODNs were 5'-end labeled with γ^{32}P-dATP and T4 polynucleotide kinase (New England Biolabs). Unincorporated label was separated from radiolabeled species by centrifugation on Chromaspin-10 columns (Clontech).

In vitro transcript and cleavage experiments. A ^{32}P-labelled 206 nt NGFI-A RNA transcript was prepared by in vitro transcription (T3 polymerase) of plasmid construct pJDM8 (as described in Milbrandt, 1987, the entire contents of which are incorporated herein by reference) previously cut with *Bgl* II. Reactions were performed in a total volume of 20 μl containing 10 mM $MgCl_2$, 5 mM Tris pH 7.5, 150 mM NaCl, 4.8 pmol of in vitro transcribed or synthetic RNA substrate and 60 pmol DNAzyme (1:12.5 substrate to DNAzyme ratio), unless otherwise indicated. Reactions were allowed to proceed at 37 °C for the times indicated and quenched by transferring an aliquot to tubes containing formamide loading buffer (Sambrook et al, 1989). Samples were run on 12% denaturing polyacrylamide gels and autoradiographed overnight at -80 °C.

Culture conditions and DNAzyme transfection. Primary rat aortic SMCs were obtained from Cell Applications, Inc., and grown in Waymouth's medium, pH 7.4, containing 10% fetal bovine serum (FBS), 50 μg/ml streptomycin and 50 IU/ml penicillin at 37 °C in a humidified atmosphere of 5% CO_2. SMCs were used in experiments between passages 3-7. Pup rat SMCs (WKY12-22 (as described in Lemire et al, 1994, the entire contents of which are incorporated herein by reference)) were grown under similar conditions. Subconfluent (60-70%) SMCs were incubated in serum-free medium (SFM) for 6 h prior to DNAzyme (or antisense ODN, where indicated) transfection (0.1 μM) using Superfect in accordance with manufacturer's instructions (Qiagen). After 18 h, the cells were washed with

phosphate-buffered saline (PBS), pH 7.4 prior to transfection a second time in 5% FBS.

Northern blot analysis. Total RNA was isolated using the TRIzol reagent (Life Technologies) and 25 μg was resolved by electrophoresis prior to transfer to Hybond-N+ membranes (NEN-DuPont). Prehybridization, hybridization with α^{32}P-dCTP-labeled Egr-1 or β-Actin cDNA, and washing was performed essentially as previously described (Khachigian et al, 1995).

Western blot analysis. Growth-quiescent SMCs in 100 mm plates (Nunc-InterMed) were transfected with ED5 or ED5SCR as above, and incubated with 5% FBS for 1 h. The cells were washed in cold PBS, pH 7.4, and extracted in 150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 1% sodium deoxycholate, 0.1% SDS, 1% Triton X-100, 5 mM EDTA, 1% trasylol, 10 μg/ml leupeptin, 1% aprotinin and 2 mM PMSF. Twenty four μg protein samples were loaded onto 10% denaturing SDS-polyacrylamide gels and electroblotted onto PVDF nylon membranes (NEN-DuPont). Membranes were air dried prior to blocking with non-fat skim milk powder in PBS containing 0.05% (w:v) Tween 20. Membranes were incubated with rabbit antibodies to Egr-1 or Sp1 (Santa Cruz Biotechnology, Inc.) (1:1000) then with HRP-linked mouse anti-rabbit Ig secondary antiserum (1:2000). Where mouse monoclonal c-Fos (Santa Cruz Biotechnology, Inc.) was used, detection was achieved with HRP-linked rabbit anti-mouse Ig. Proteins were visualized by chemiluminescent detection (NEN-DuPont).

Assays of cell proliferation. Growth-quiescent SMCs in 96-well titer plates (Nunc-InterMed) were transfected with ED5 or ED5SCR as above, then exposed to 5% FBS at 37 °C for 72 h. The cells were rinsed with PBS, pH 7.4, trypsinized and the suspension was quantitated using an automated Coulter counter.

Assessment of DNAzyme stability. DNAzymes were 5'-end labeled with γ^{32}P-dATP and separated from free label by centrifugation. Radiolabeled DNAzymes were incubated in 5% FBS or serum-free medium at 37 °C for the times indicated. Aliquots of the reaction were quenched by transfer to tubes containing formamide loading buffer (Sambrook et al, 1989). Samples were applied to 12% denaturing polyacrylamide gels and autoradiographed overnight at -80 °C.

SMC wounding assay. Confluent growth-quiescent SMCs in chamber slides (Nunc-InterMed) were exposed to ED5 or ED5SCR for 18 h prior to a

single scrape with a sterile toothpick. Cells were treated with mitomycin C (Sigma) (20 μM) for 2 h prior to injury (Pitsch et al, 1996; Horodyski & Powell, 1996). Seventy-two h after injury, the cells were washed with PBS, pH 7.4, fixed with formaldehyde then stained with hematoxylin-eosin.

Rat arterial ligation model and analysis. Adult male Sprague Dawley rats weighing 300-350 g were anaesthetised using ketamine (60 mg/kg, i.p.) and xylazine (8 mg/kg, i.p.). The right common carotid artery was exposed up to the carotid bifurcation via a midline neck incision. Size 6/0 non-absorbable suture was tied around the common carotid proximal to the bifurcation, ensuring cessation of blood flow distally. A 200 μl solution at 4°C containing 500 μg of DNAzyme (in DEPC-treated H_2O), 1mM $MgCl_2$, 30 μl of transfecting agent (Fugene 6) and Pluronic gel P127 (BASF) was applied around the vessel in each group of 5 rats, extending proximally from the ligature for 12-15 mm. These agents did not inhibit the solidification of the gel at 37 °C. After 3 days, vehicle with or without 500 μg of DNAzyme was administered a second time. Animals were sacrificed 18 days after ligation by lethal injection of phenobarbitone, and perfusion fixed using 10% (v:v) formaldehyde perfused at 120 mm Hg. Both carotids were then dissected free and placed in 10% formaldehyde, cut in 2 mm lengths and embedded in 3% (w:v) agarose prior to fixation in paraffin. Five μm sections were prepared at 250 μm intervals along the vessel from the point of ligation and stained with hematoxylin and eosin. The neointimal and medial areas of 5 consecutive sections per rat were determined digitally using a customized software package (Magellan) (Halasz & Martin, 1984) and expressed as a mean ratio per group of 5 rats.

Results and Discussion

The 7x7 nt arms flanking the 15 nt DNAzyme catalytic domain in the original DNAzyme design (Santoro and Joyce, 1997) were extended by 2 nts per arm for improved specificity (L.-Q. Sun, data not shown) (Figure 6). The 3' terminus of the molecule was capped with an inverted 3'-3'-linked thymidine (T) to confer resistance to 3'->5' exonuclease digestion. The sequence in both arms of ED5 was scrambled (SCR) without altering the catalytic domain to produce DNAzyme ED5SCR (Figure 6).

A synthetic RNA substrate comprised of 23 nts, matching nts 805 to 827 of NGFI-A mRNA (Figure 6) was used to determine whether ED5 had the

capacity to cleave target RNA. ED5 cleaved the ^{32}P-5'-end labeled 23-mer within 10 min (data not shown). The 12-mer product corresponds to the length between the A(816)-U(817) junction and the 5' end of the substrate (Figure 6). In contrast, ED5SCR had no demonstrable effect on this synthetic substrate. Specific ED5 catalysis was further demonstrated by the inability of the human equivalent of this DNAzyme (hED5) to cleave the rat substrate over a wide range of stoichiometric ratios (data not shown). Similar results were obtained using ED5SCR (data not shown). hED5 differs from the rat ED5 sequence by 3 of 18 nts in its hybridizing arms (Table 2). The catalytic effect of ED5 on a ^{32}P-labeled 206 nt fragment of native NGFI-A mRNA prepared by *in vitro* transcription was then determined. The cleavage reaction produced two radiolabeled species of 163 and 43 nt length consistent with DNAzyme cleavage at the A(816)-U(817) junction. In other experiments, ED5 also cleaved a ^{32}P-labeled NGFI-A transcript of 1960 nt length in a specific and time-dependent manner (data not shown).

Table 2. DNAzyme target sites in mRNA.

Similarity between the 18 nt arms of ED5 or hED5 and the mRNA of rat NGFI-A or human EGR-1 (among other transcription factors) is expressed as a percentage. The target sequence of ED5 in NGFI-A mRNA is 5'-807-**A** CGU CCG GGA UGG CAG CGG-825-3' (SEQ ID NO: 22) (rat NGFI-A sequence), and that of hED5 in EGR-1 is 5'-262-**U** CGU CCA GGA UGG CCG CGG-280-3' (SEQ ID NO: 23) (Human EGR-1 sequence). Nucleotides in bold indicate mismatches between rat and human sequences. Data obtained by a gap best fit search in ANGIS using sequences derived from Genbank and EMBL. Rat sequences for Sp1 and c-Fos have not been reported.

Gene	Accession number	Best homology over 18 nts (%)	
		ED5	hED5
Rat NGFI-A	M18416	**100**	84.2
Human EGR-1	X52541	84.2	**100**
Murine Sp1	AF022363	66.7	66.7
Human c-Fos	K00650	66.7	66.7
Murine c-Fos	X06769	61.1	66.7
Human Sp1	AF044026	38.9	28.9

To determine the effect of the DNAzymes on endogenous levels of NGFI-A mRNA, growth-quiescent SMCs were exposed to ED5 prior to stimulation with serum. Northern blot and densitometric analysis revealed that ED5 (0.1 μM) inhibited serum-inducible steady-state NGFI-A mRNA levels by 55% (data not shown), whereas ED5SCR had no effect (data not shown). The capacity of ED5 to inhibit NGFI-A synthesis at the level of protein was assessed by Western blot analysis. Serum-induction of NGFI-A protein was suppressed by ED5. In contrast, neither ED5SCR nor EDC, a DNAzyme bearing an identical catalytic domain as ED5 and ED5SCR but flanked by nonsense arms had any influence on the induction of NGFI-A

(Figure 7). ED5 failed to affect levels of the constitutively expressed, structurally-related zinc-finger protein, Sp1 (Figure 7). It was also unable to block serum-induction of the immediate-early gene product, c-Fos (Figure 7) whose induction, like NGFI-A, is dependent upon serum response elements in its promoter and phosphorylation mediated by extracellular-signal regulated kinase (Treisman, 1990, 1994 and 1995; Gashler & Sukhatme, 1995). These findings, taken together, demonstrate the capacity of ED5 to inhibit production of NGFI-A mRNA and protein in a gene-specific and sequence-specific manner, consistent with the lack of significant homology between its target site in NGFI-A mRNA and other mRNA (Table 2).

The effect of ED5 on SMC replication was next determined. Growth-quiescent SMCs were incubated with DNAzyme prior to exposure to serum and the assessment of cell numbers after 3 days. ED5 (0.1 μM) inhibited SMC proliferation stimulated by serum by 70% (Figure 8a). In contrast, ED5SCR failed to influence SMC growth (Figure 8a). AS2, an antisense NGFI-A ODN able to inhibit SMC growth at 1 μM failed to inhibit proliferation at the lower concentration (Figure 8a). Additional experiments revealed that ED5 also blocked serum-inducible ^{3}H-thymidine incorporation into DNA (data not shown). ED5 inhibition was not a consequence of cell death since no change in morphology was observed, and the proportion of cells incorporating Trypan Blue in the presence of serum was not influenced by either DNAzyme (Figure 8b).

Cultured SMCs derived from the aortae of 2 week-old rats (WKY12-22) are morphologically and phenotypically similar to SMCs derived from the neointima of balloon-injured rat arteries (Seifert et al, 1984; Majesky et al, 1992). The epitheloid appearance of both WKY12-22 cells and neointimal cells contrasts with the elongated, bipolar nature of SMCs derived from normal quiescent media (Majesky et al, 1988). WKY12-22 cells grow more rapidly than medial SMCs and overexpress a large number of growth-regulatory molecules (Lemire et al, 1994), such as NGFI-A (Rafty & Khachigian, 1998), consistent with a "synthetic" phenotype (Majesky et al, 1992; Campbell & Campbell, 1985). ED5 attenuated serum-inducible WKY12-22 proliferation by approximately 75% (Figure 8c). ED5SCR had no inhibitory effect; surprisingly, it appeared to stimulate growth (Figure 8c). Trypan Blue exclusion revealed that DNAzyme inhibition was not a consequence of cytotoxicity (data not shown).

To ensure that differences in the biological effects of ED5 and ED5SCR were not the consequence of dissimilar intracellular localization, both DNAzymes were 5'-end labeled with fluorescein isothiocyanate (FITC) and incubated with SMCs. Fluorescence microscopy revealed that both FITC-ED5 and FITC-ED5SCR localized mainly within the nuclei. Punctate fluorescence in this cellular compartment was independent of DNAzyme sequence. Fluorescence was also observed in the cytoplasm, albeit with less intensity. Cultures not exposed to DNAzyme showed no evidence of autofluorescence.

Both molecules were 5'-end labeled with γ^{32}P-dATP and incubated in culture medium to ascertain whether cellular responsiveness to ED5 and ED5SCR was a consequence of differences in DNAzyme stability. Both ^{32}P-ED5 and ^{32}P-ED5SCR remained intact even after 48 h (data not shown). In contrast to ^{32}P-ED5 bearing the 3' inverted T, degradation of ^{32}P-ED5 bearing its 3' T in the correct orientation was observed as early as 1 h. Exposure to serum-free medium did not result in degradation of the molecule even after 48 h (data not shown). These findings indicate that inverse orientation of the 3' base in the DNAzyme protects the molecule from nucleolytic cleavage by components in serum.

Physical trauma imparted to SMCs in culture results in outward migration from the wound edge and proliferation in the denuded zone. We determined whether ED5 could modulate this response to injury by exposing growth-quiescent SMCs to either DNazyme and Mitomycin C, an inhibitor of proliferation (Pitsch et al, 1996; Horodyski & Powell, 1996) prior to scraping. Cultures in which DNAzyme was absent repopulated the entire denuded zone within 3 days. ED5 inhibited this reparative response to injury and prevented additional growth in this area even after 6 days (data not shown). That ED5SCR had no effect in this system further demonstrates sequence-specific inhibition by ED5.

The effect of ED5 on neointima formation was investigated in a rat model. Complete ligation of the right common carotid artery proximal to the bifurcation results in migration of SMCs from the media to the intima where proliferation eventually leads to the formation of a neointima (Kumar & Lindner, 1997; Bhawan et al, 1977; Buck, 1961). Intimal thickening 18 days after ligation was inhibited 50% by ED5 (Figure 9). In contrast, neither its scrambled counterpart (Figure 9) nor the vehicle control (Figure 9) had any

effect on neointima formation. These findings demonstrate the capacity of ED5 to suppress SMC accumulation in the vascular lumen in a specific manner, and argue against inhibition as a mere consequence of a "mass effect" (Kitze et al, 1998; Tharlow et al, 1996). Sequence specific inhibition of inducible NGFI-A protein expression and intimal thickening by ED5 was also observed in the rat carotid balloon injury model (Santiago et al., 1999a).

Further experiments revealed the capacity of hED5 to cleave (human) EGR-1 RNA. hED5 cleaved its substrate in a dose-dependent manner over a wide range of stoichiometric ratios. hED5 also cleaved in a time-dependent manner, whereas hED5SCR, its scrambled counterpart, had no such catalytic property (data not shown).

The specific, growth-inhibitory properties of antisense EGR-1 strategies reported herein suggest that EGR-1 inhibitors may be useful as therapeutic tools in the treatment of vascular disorders involving inappropriate SMC growth, endothelial growth and tumour growth.

EXAMPLE 3

Use of DNAzymes to inhibit growth of malignant cells

Materials and Methods

HepG2 cells were routinely grown in DMEM, pH 7.4, containing 10 % fetal calf serum supplemented with antibiotics. The cells were trypsinized, resuspended in growth medium (to 10,000 cells/200 μl) and 200 μl transferred into sterile 96 well titre plates. Two days subsequently, 180 μl of the culture supernatant was removed, the cells were washed with PBS, pH 7.4, and refed with 180 μl of serum free media. After 6 h, the first transfection of DNAzyme (2 μg/200μl wall, 0.75 μM final) was performed in tubes containing serum free media using FuGENE6 at a ratio of 1:3 (μg:μl). After 15 min incubation at room temperature, 180 μl of the culture supernantant was replaced with 180 μl of the transfection mix. After 24 h, 180μl of the supernatant was replaced with 180 μl of new transfection mix, but this time in 5% FBS media. After 3 days, the cells were washed in PBS, pH 7.4, and resuspended by trypsinization in 100 μl trypsin-EDTA. The cells were shaken for approximately 5 min to ensure the cells were in suspension. The entire suspension was placed into 10 ml of Isoton II. That all the cells were transferred was ensured by pipetting Isoton II solution from tubes back into wells several times. Using Isoton II only, background cell

number was determined. Each sample was counted three times and used to calculate mean counts and standard errors of each mean.

Results and Discussion

Our results indicate that serum stimulated HepG2 cell proliferation after 3 days (Figure 10). Proliferation was almost completely suppressed by 0.75 μM of DzA (5'-caggggacaGGCTAGCTACAACGAcgttgcggg (SEQ ID NO:3), catalytic moiety in capitals), a DNAzyme targeting human EGR-1 mRNA (arms hybridize to nts 189-207) (Figure 10). In contrast, HepG2 cell growth was not inhibited by ED5SCR (Figure 10). Western blot analysis revealed that DzA strongly inhibited EGR-1 expression in HepG2 cells, whereas a size matched DNAzyme with different sequence (5'-tcagctgcaGGCTAGCTACAACGActcggcctt) (SEQ ID NO:24) had no effect (data not shown). These data indicate that inducible proliferation of this model human malignant cell line can be blocked by the EGR-1 DNAzyme. These findings suggest that EGR inhibitors may be clinically useful in therapeutic strategies targeting human cancer.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive. Throughout this application, various publications are cited. The disclosure of these publications is hereby incorporated by reference into this application to describe more fully the state of the art to which the invention pertains.

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Claims:

1. A method for the treatment of a tumour, the method comprising administering to a subject in need thereof an agent which inhibits induction of an EGR, an agent which decreases expression of an EGR or an agent which decreases the nuclear accumulation or activity of an EGR.

2. A method as claimed in claim 1 in which the agent inhibits angiogenesis.

3. A method as claimed in claim 1 or claim 2 in which the agent directly inhibits proliferation of the tumour cells.

4. A method as claimed in any one of claims 1 to 3 in which the tumour is a solid tumour.

5. A method as claimed in any one of claims 1 to 4 in which the EGR is EGR-1.

6. A method as claimed in any one of claims 1 to 5 in which the expression of EGR is decreased.

7. A method as claimed in claim 6 in which the expression of EGR is decreased by the use of an EGR antisense oligonucleotide.

8. A method as claimed in claim 7 in which the antisense oligonucleotide has a sequence selected from the group consisting of

(i) ACA CTT TTG TCT GCT (SEQ ID NO:4), and

(ii) CTT GGC CGC TGC CAT (SEQ ID NO:2).

9. A method as claimed in claim 6 in which the expression of EGR is decreased by the cleavage of EGR mRNA by a sequence-specific ribozyme.

10. A method as claimed in claim 6 in which the expression of EGR is decreased by the use of a ssDNA targeted against EGR dsDNA the ssDNA molecule being selected so as to form a triple helix with the dsDNA.

11. A method as claimed claim 6 in which the expression of EGR is decreased by inhibiting transcription of the EGR gene using a nucleic acid transcriptional decoy.

12. A method as claimed in claim 6 in which the expression of EGR is decreased by the expression of antisense EGR mRNA .

13. A method as claimed in claim 6 in which the expression of EGR is decreased by cleavage of EGR mRNA by a sequence specific DNAzyme.

14. A method as claimed in claim 13 in which the DNAzyme comprises

(i) a catalytic domain which cleaves mRNA at a purine:pyrimidine cleavage site;

(ii) a first binding domain contiguous with the 5' end of the catalytic domain; and

(iii) a second binding domain contiguous with the 3' end of the catalytic domain,

wherein the binding domains are sufficiently complementary to two regions immediately flanking a purine:pyrimidine cleavage site within the region of EGR mRNA corresponding to nucleotides 168 to 332 as shown in SEQ ID NO:15, such that the DNAzyme cleaves the EGR mRNA.

15. A method as claimed in claim 13 or claim 14 in which the catalytic domain has the nucleotide sequence GGCTAGCTACAACGA.

16. A method as claimed in any one of claims 13 to 15 in which the cleavage site is selected from the group consisting of

(i) the GU site corresponding to nucleotides 198-199;

(ii) the GU site corresponding to nucleotides 200-201;

(iii) the GU site corresponding to nucleotides 264-265;

(iv) the AU site corresponding to nucleotides 271-272;

(v) the AU site corresponding to nucleotides 301-302;

(vi) the GU site corresponding to nucleotides 303-304; and

(vii) the AU site corresponding to nucleotides 316-317.

17. A method as claimed in claim 16 in which the cleavage site is the GU site corresponding to nucleotides 198-199, the AU site corresponding to nucleotides 271-272 or the AU site corresponding to nucleotides 301-302.

18. A method as claimed in claim 16 in which the DNAzyme has a sequence selected from the group consisting of:

(i) 5'-caggggacaGGCTAGCTACAACGAcgttgcggg (SEQ ID NO:3);

(ii) 5'-tgcaggggaGGCTAGCTACAACGAaccgttgcg (SEQ ID NO:6);

(iii) 5'-catcctggaGGCTAGCTACAACGAgagcaggct (SEQ ID NO:7);

(iv) 5'-ccgcggccaGGCTAGCTACAACGAcctggacga (SEQ ID NO:8);

(v) 5'-ccgctgccaGGCTAGCTACAACGAcccggacgt (SEQ ID NO:9);

(vi) 5'-gcggggacaGGCTAGCTACAACGAcagctgcat (SEQ ID NO:10);

(vii) 5'-cagcggggaGGCTAGCTACAACGAatcagctgc (SEQ ID NO:11); and

(viii) 5'-ggtcagagaGGCTAGCTACAACGActgcagcgg (SEQ ID NO:12).

19. A method as claimed in claim 18 in which the DNAzyme has the sequence: 5'-caggggacaGGCTAGCTACAACGAcgttgcggg (SEQ ID NO:3) or 5'-gcggggacaGGCTAGCTACAACGAcagctgcat (SEQ ID NO:10).

20. A method as claimed in claim 18 in which the DNAzyme has the sequence: 5'-ccgcggccaGGCTAGCTACAACGAcctggacga (SEQ ID NO:8) or 5'-ccgctgccaGGCTAGCTACAACGAcccggacgt (SEQ ID NO:9).

21. A method as claimed in any one of claims 13 to 19, wherein the 3'-end nucleotide residue of the DNAzyme is inverted in the binding domain contiguous with the 3' end of the catalytic domain.

22. A method as claimed in any one of claims 1 to 21 which further comprises administering one or more additional anti-cancer agents.

23. A method for inhibiting the growth or proliferation of a tumour cell, the method comprising contacting a tumour cell with an agent which inhibits induction of EGR, an agent which decreases expression of EGR or an agent which decreases the nuclear accumulation or activity of EGR.

24. A tumour cell which has been transformed by introducing into the cell a nucleic acid molecule, the nucleic acid molecule comprising or encoding (i) an agent which inhibits induction of EGR, (ii) an agent which decreases expression of EGR, or (iii) an agent which decreases the nuclear accumulation or activity of EGR.

25. A method of screening for an agent which inhibits angiogenesis, the method comprising testing a putative agent for the ability to inhibit induction of EGR, decrease expression of EGR or decrease the nuclear accumulation or activity of EGR.

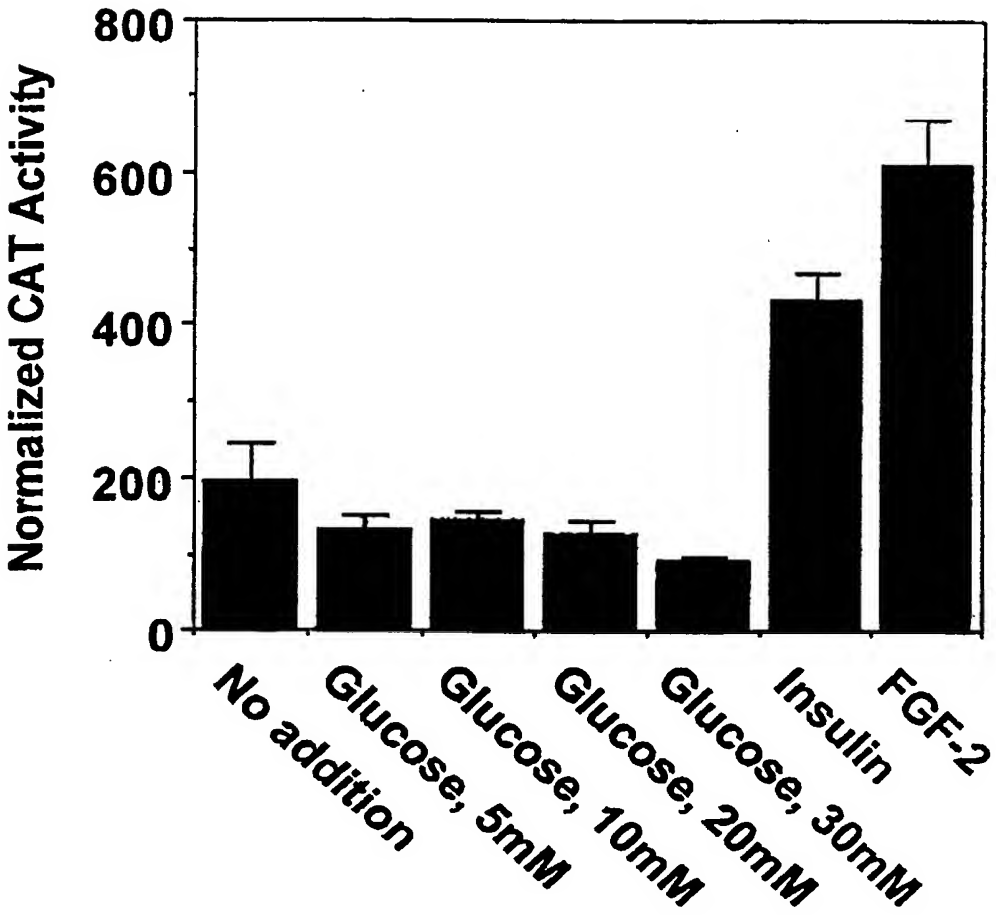

Normalized CAT Activity
800
600
400
200
0
No addition
Glucose, 5mM
Glucose, 10mM
Glucose, 20mM
Glucose, 30mM
Insulin
FGF-2

Figure 1

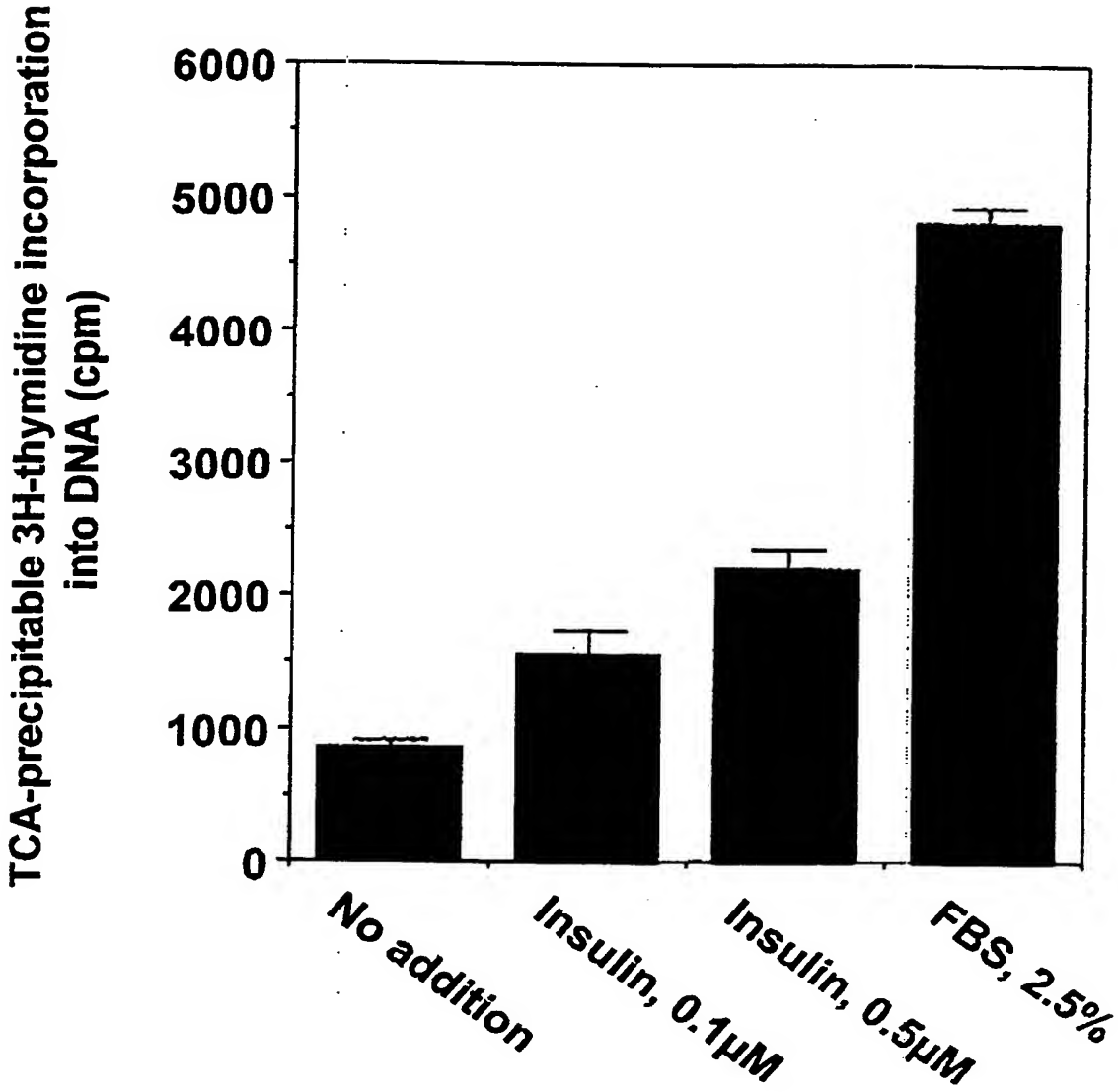

TCA-precipitable 3H-thymidine incorporation into DNA (cpm)
6000
5000
4000
3000
2000
1000
0
No addition
Insulin, 0.1µM
Insulin, 0.5µM
FBS, 2.5%

Figure 2A

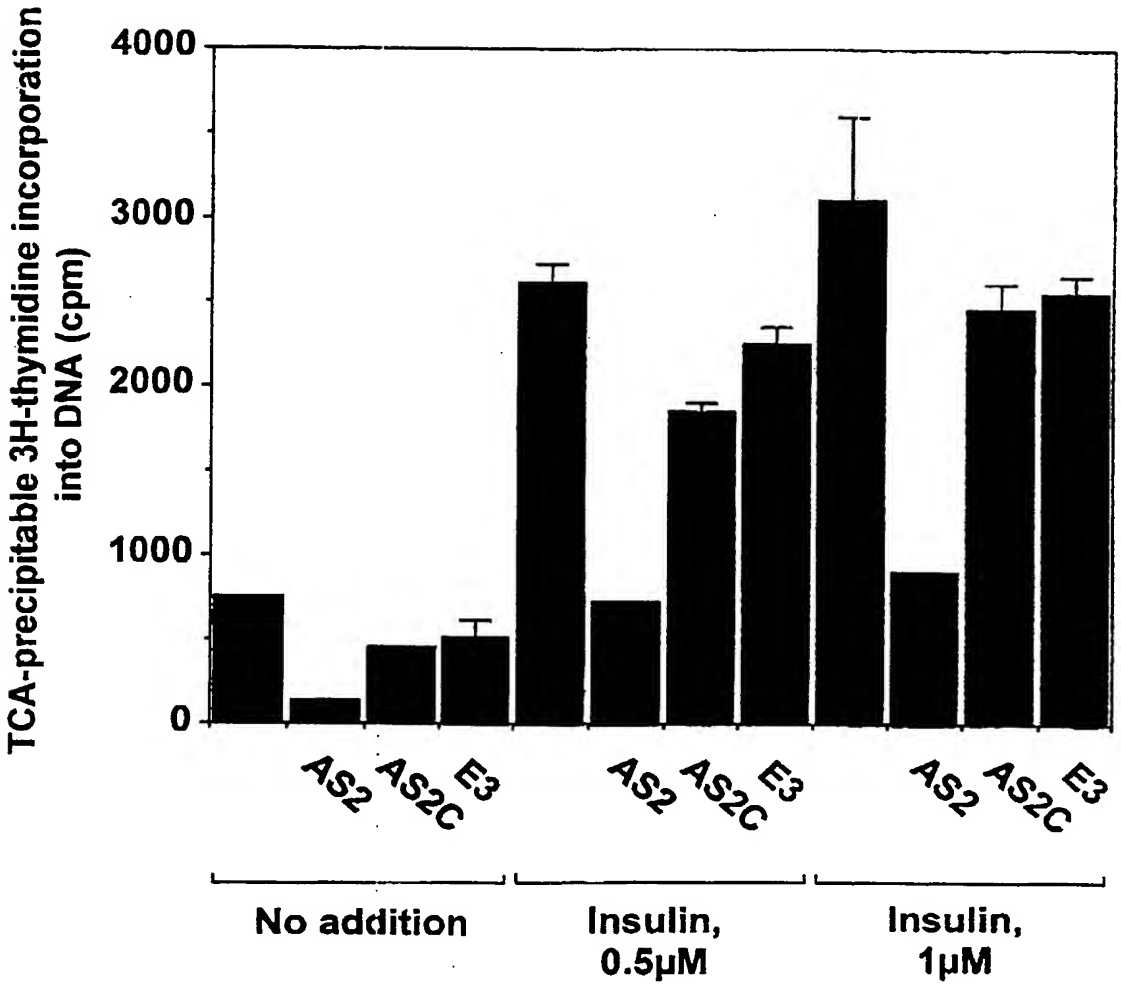

TCA-precipitable 3H-thymidine incorporation into DNA (cpm)
4000
3000
2000
1000
0
AS2
AS2C
E3
AS2
AS2C
E3
AS2
AS2C
E3
No addition
Insulin, 0.5μM
Insulin, 1μM

Figure 2B

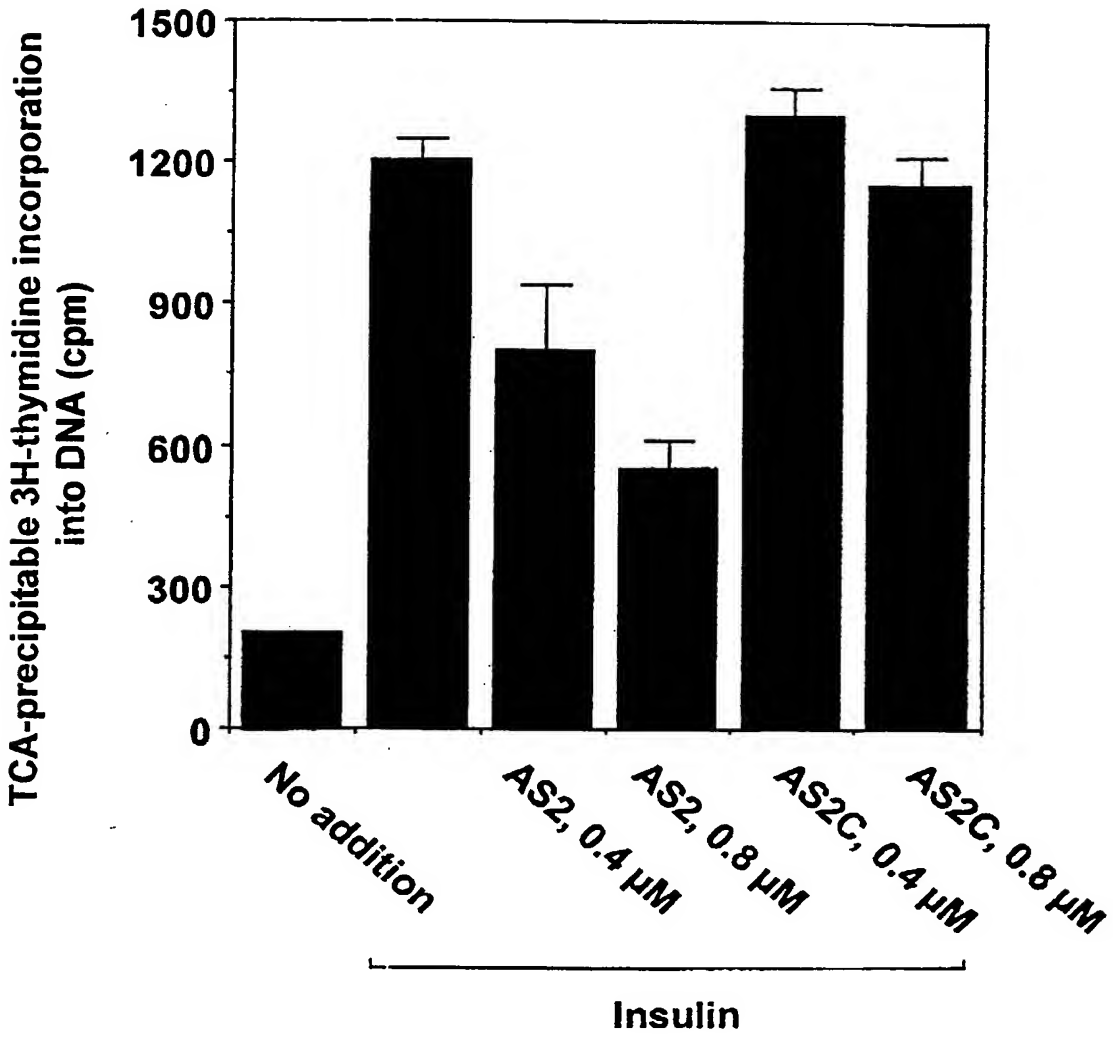

TCA-precipitable 3H-thymidine incorporation into DNA (cpm)
1500
1200
900
600
300
0
No addition
AS2, 0.4 µM
AS2, 0.8 µM
AS2C, 0.4 µM
AS2C, 0.8 µM
Insulin

Figure 2C

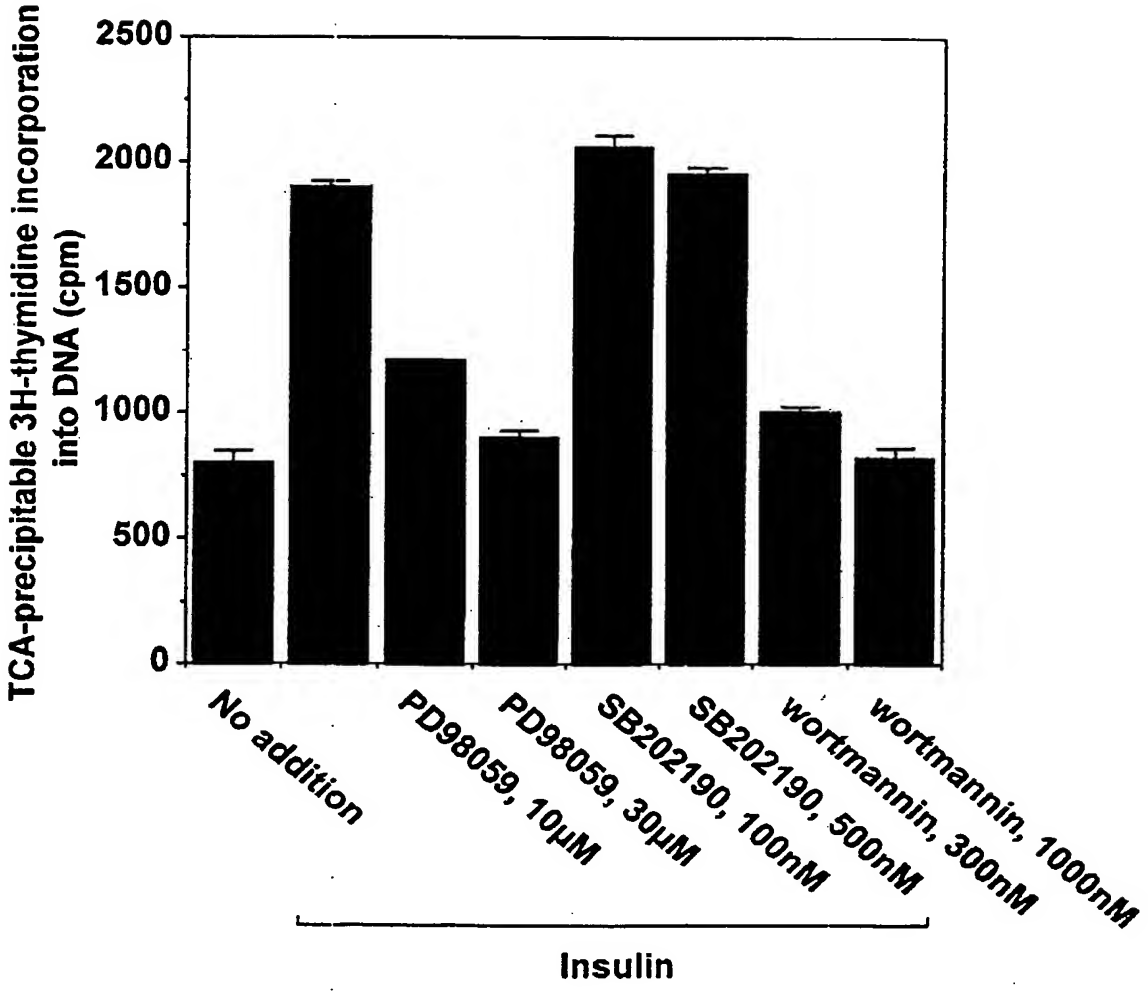

TCA-precipitable 3H-thymidine incorporation into DNA (cpm)
2500
2000
1500
1000
500
0
No addition
PD98059, 10μM
PD98059, 30μM
SB202190, 100nM
SB202190, 500nM
wortmannin, 300nM
wortmannin, 1000nM
Insulin

Figure 3

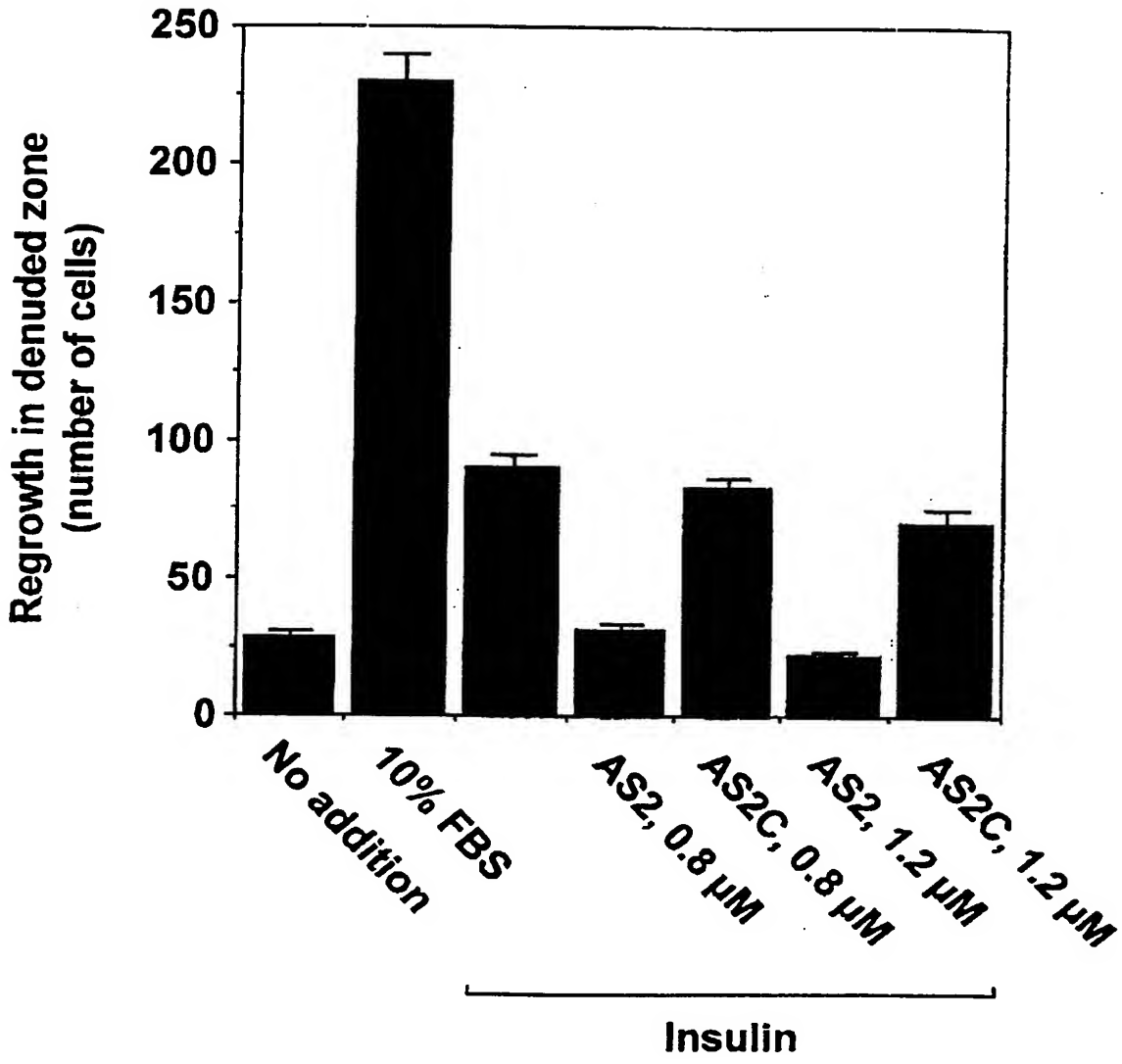

Regrowth in denuded zone
(number of cells)
250
200
150
100
50
0
No addition
10% FBS
AS2, 0.8 µM
AS2C, 0.8 µM
AS2, 1.2 µM
AS2C, 1.2 µM
Insulin

Figure 4

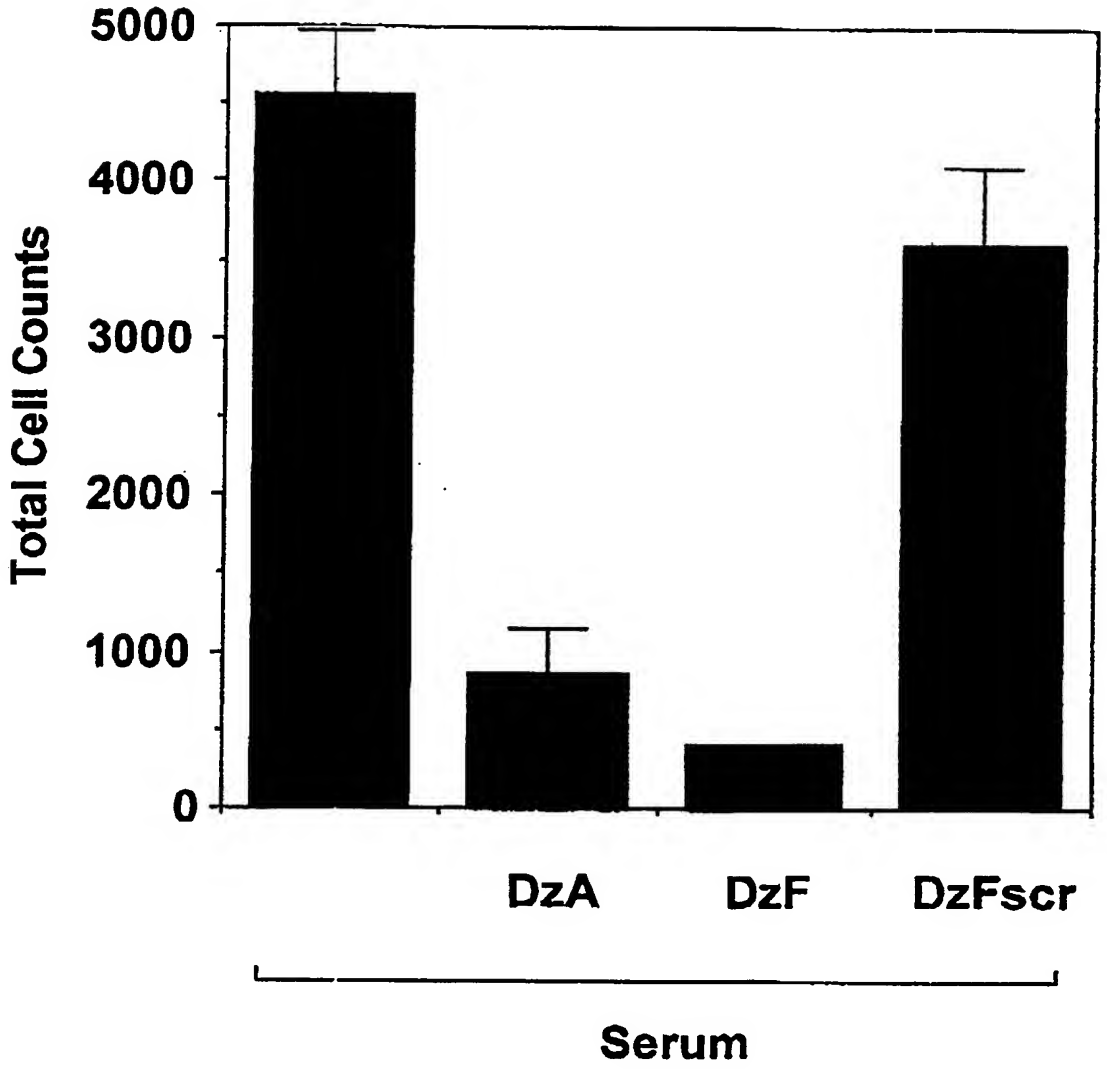
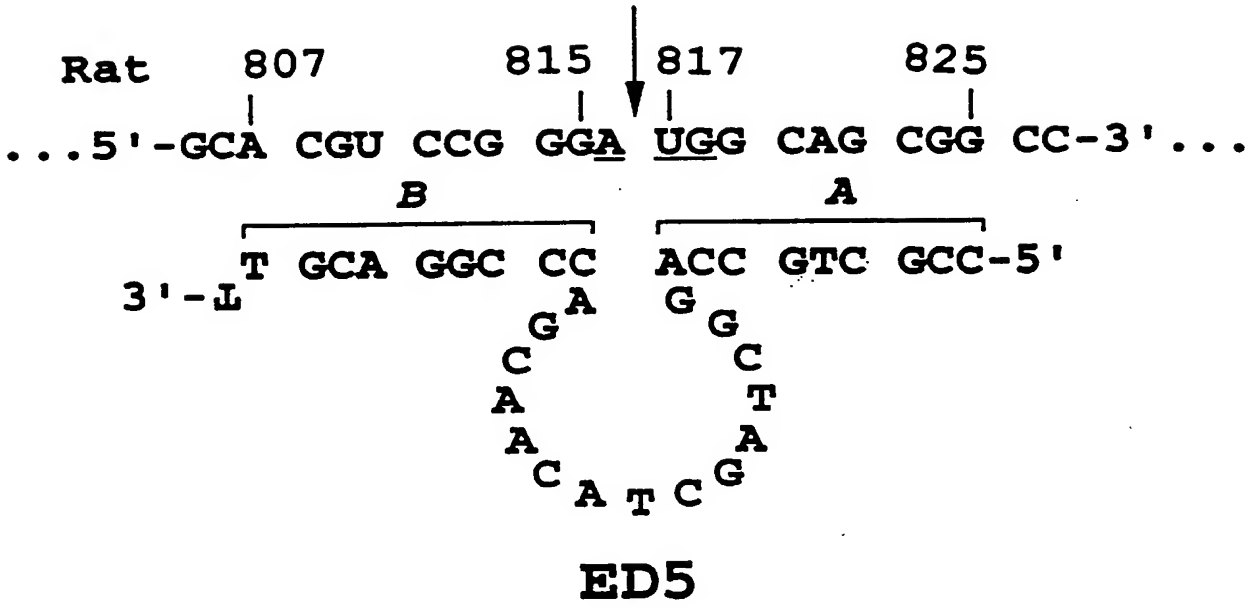
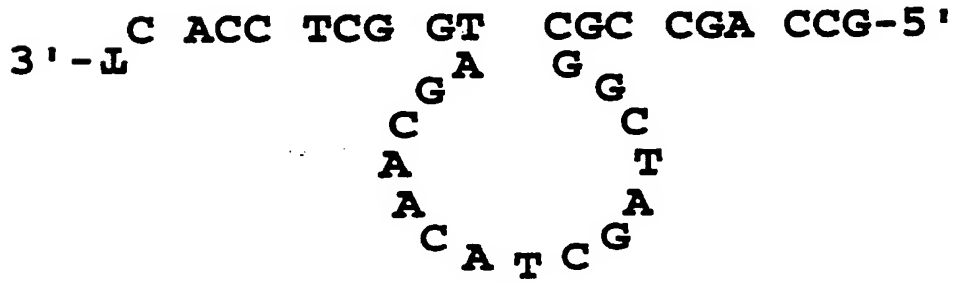

5000
4000
3000
2000
1000
0
Total Cell Counts
DzA
DzF
DzFscr
Serum

Figure 5



ED5



ED5SCR

Figure 6

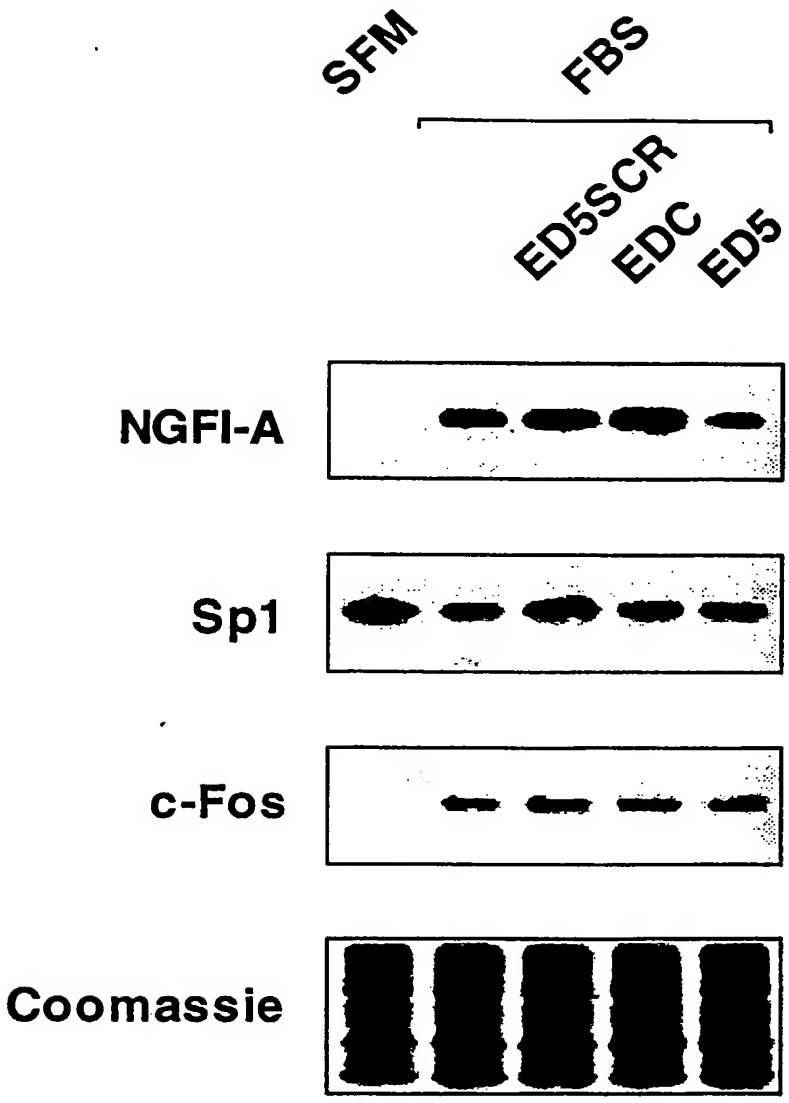


Figure 7

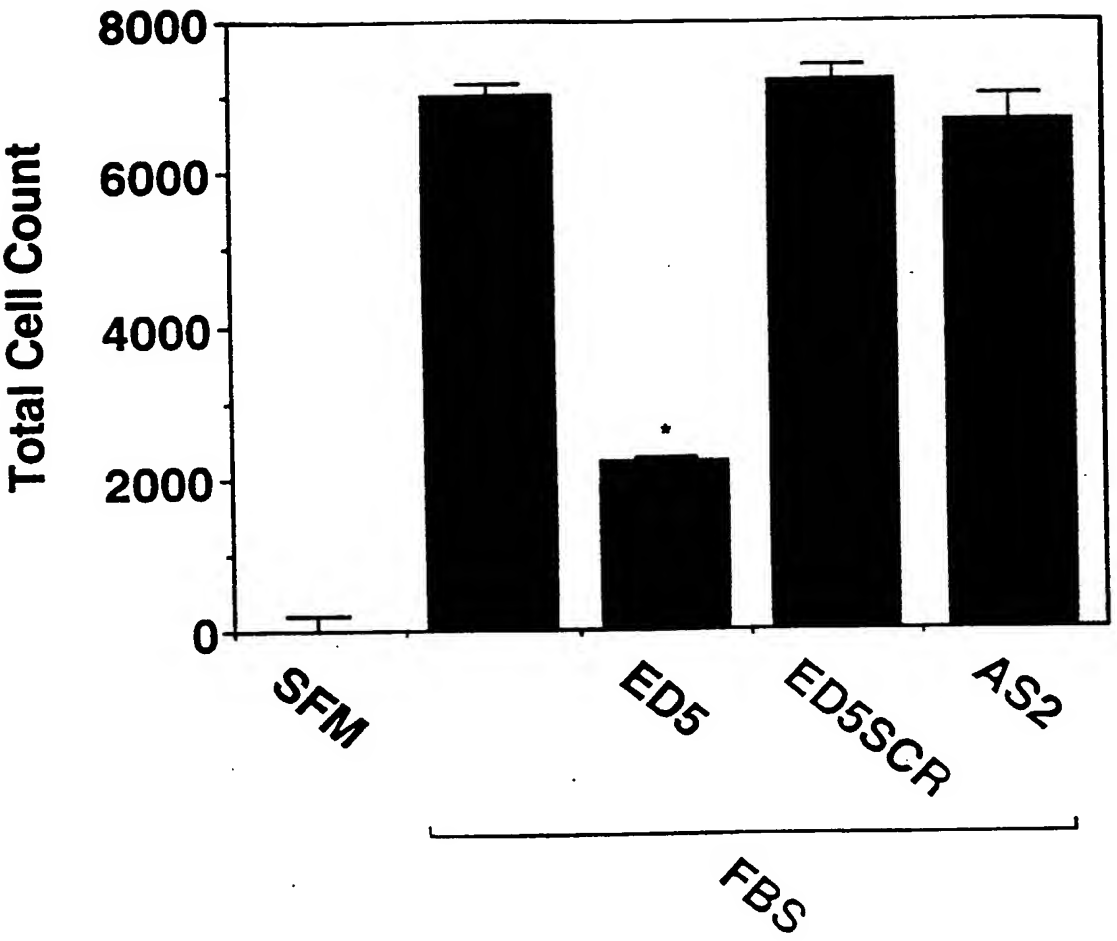


Figure 8A

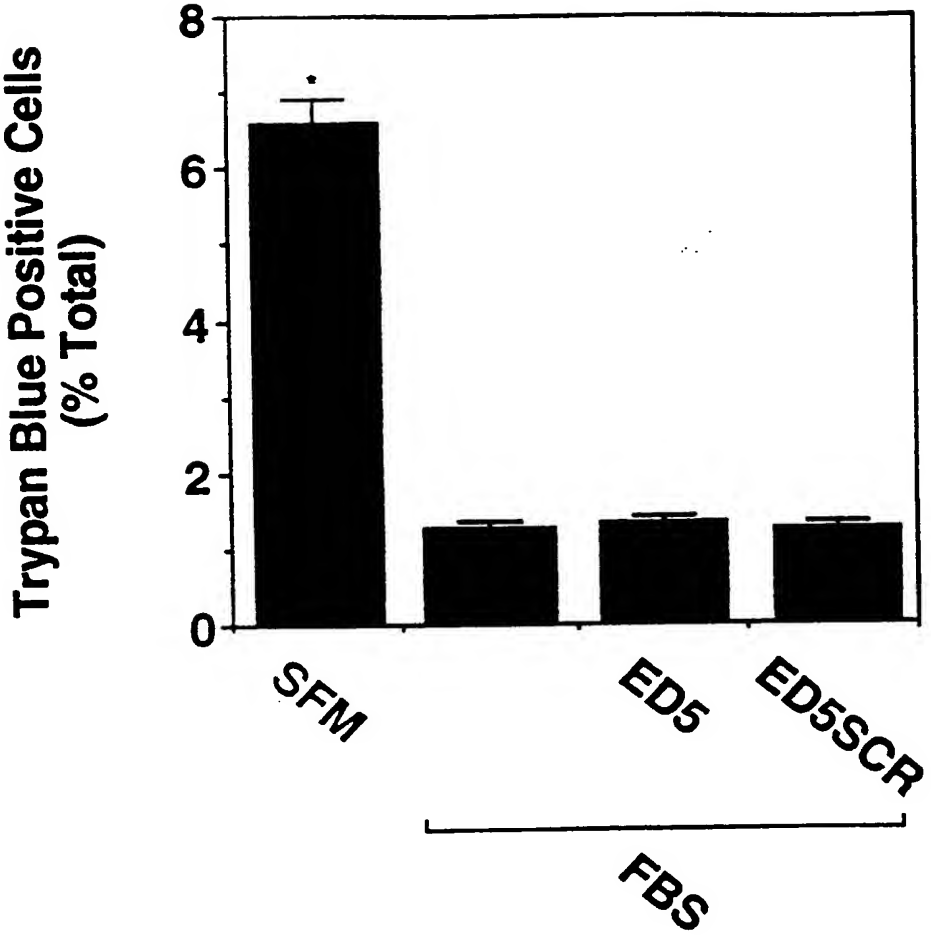
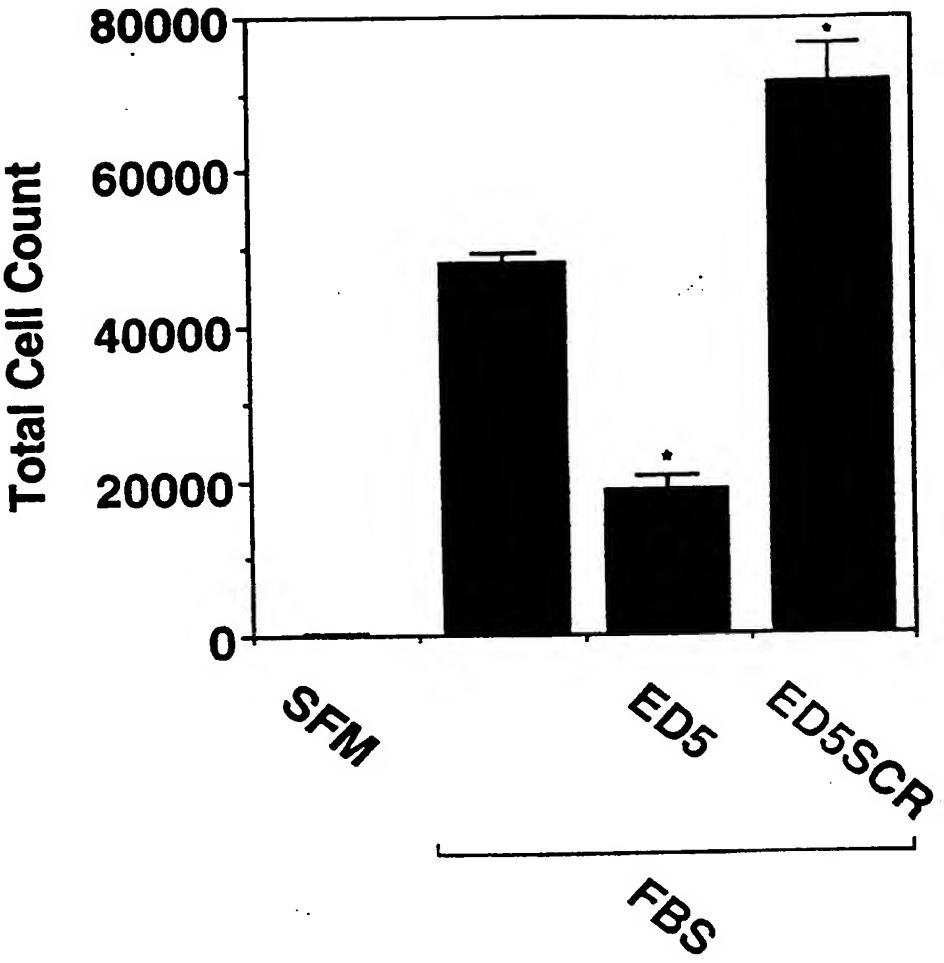


Figure 8B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60000
40000
20000
0
Total Cell Count
SFM
ED5
ED5SCR
FBS

Figure 8C

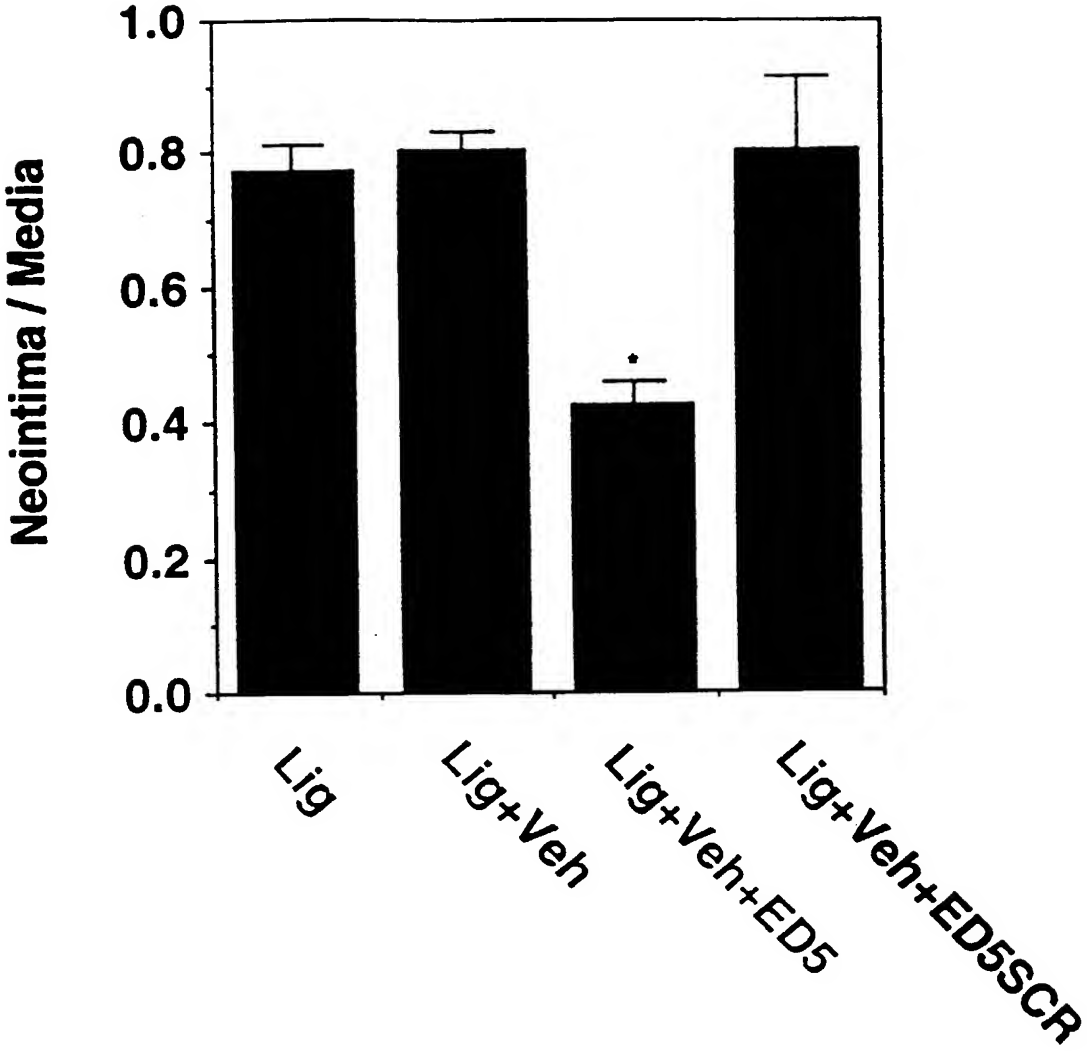
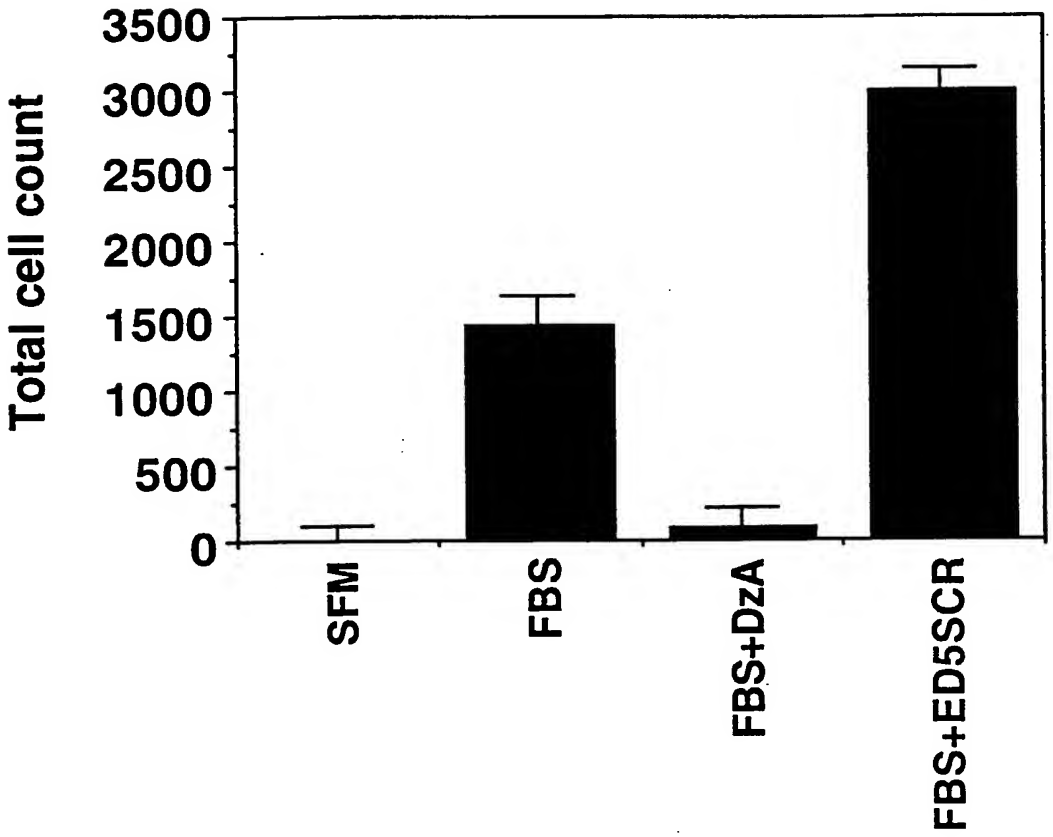

Neointima / Media
1.0
0.8
0.6
0.4
0.2
0.0
*
Lig
Lig+Veh
Lig+Veh+ED5
Lig+Veh+ED5SCR

Figure 9


Total cell count
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3000
2500
2000
1500
1000
500
0
SFM
FBS
FBS+DzA
FBS+ED5SCR

Figure 10

SEQUENCE LISTING

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<120> Treatment of cancer

<160> 24

<170> PatentIn Ver. 2.1

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<220>
<223> Description of Artificial Sequence: DNAzyme

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<212> DNA
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<220>
<223> Description of Artificial Sequence: antisense oligonucleotide

<400> 2
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<210> 3
<211> 33
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: DNAzyme

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<212> DNA
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<220>
<223> Description of Artificial Sequence:antisense oligonucleotide

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acacttttgt ctgct 15

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<220>
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<400> 5
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<210> 6
<211> 33
<212> DNA
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<220>
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<220>
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<400> 8
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<223> Description of Artificial Sequence: DNAzyme

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/01315

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl. [7]: A61K 48/00, A61P 35/00, C12N15/11, C07H 21/04

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
DERWENT DATABASES: WPAT; CHEMICAL ABSTRACTS

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
MEDLINE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
DERWENT DATABASES: WPAT; CHEMICAL ABSTRACTS; MEDLINE - Keywords used (see Supplemental Box)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	AU, B, 20865/97 (707943) (UNISEARCH LIMITED) opi. 22 September 1997, epd. 7 March 1996 (pages 8-17 & claims)	1-13, 22-25 14-21
X Y	J. of Biological Chemistry, 268(26), 15 September 1993, 'NGFI-A Gene Expression Is Necessary for T Lymphocyte Proliferation', A. Perez-Castillo et.al., pages 19445-19450 (entire document)	1-7, 9-13, 22-25 8, 14-21
X Y	Molecular and Cellular Biology, 15(2), February 1995, 'The Zinc Finger Transcription Factor EGR-1 Impedes Interleukin-I-Inducible Tumor Growth Arrest', S.F. Sells et.al., pages 682-692 (entire document)	1-7, 9-13, 22-25 8, 14-21

[X] Further documents are listed in the continuation of Box C [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
20 December 2000	28 DEC 2000
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer KAREN TAN Telephone No : (02) 6283 2277

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/01315

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	J. of Biological Chemistry, 272(44), 31 October 1997, 'Role of *EGR*-1 Expression in B Cell Receptor-induced Apoptosis in an Immature B Cell Lymphoma', S. Muthukkumar et.al., pages 27987-27993 (entire document)	1-7, 9-13, 22-25 8, 14-21
X Y	Cardiovascular Pathobiology, 2(3/4), 1998, 'Prostaglandin $F_{2\alpha}$-induced Growth of Neonatal Rat Cardiomyocytes is Inhibited by Antisense Egr-1 Oligodeoxynucleotides', J.W. Adams and S. A. Henderson, pages 167-180 (entire document)	1, 2, 5-7, 9-13, 25 8, 14-21
X	Cancer Research, 58, 1 June 1998, 'Expression of Early Growth Response Genes in Human Prostate Cancer', M.A. Eid et. al., pages 2461-2468 (entire document)	1-7, 9-13, 22-25
X	Oncogene, 17(19), 1998, 'Wild-type egr1/Krox24 promotes and dominant-negative mutants inhibit, pluripotent differentiation of p19 embryonal carcinoma cells', J. Lanoix et.al., pages 2495-2504 (entire document)	1-6, 22-25
X Y	American Journal of Pathology, 155(3), September 1999, 'Vascular Smooth Muscle Cell Proliferation and Regrowth after Mechanical Injury *in vitro* Are Egr-1/NGFI-A-Dependent', F.S. Santiago et.al., pages 697-905 (entire document)	1, 2, 5-7, 9-13, 25 8, 14-21
Y	US 5 807 718 (JOYCE et.al.) publ. 15 September 1998, epd. 7 June 1995 (entire document)	8, 14-21
Y	Proc. Natl. Acad. Sci. USA, 94, April 1997, 'A general purpose RNA-Cleaving DNA enzyme', S.W. Santoro and G.F. Joyce, pages 4262-4266 (entire document)	8, 14-21
P, X	AU, A, 44487/00 (MERCOLA & ADAMSON) opi. 25 August 2000, epd. 5 February 1999 (entire document)	1-25
P, X	AU, A, 21444/00 (ISIS PHARMACEUTICALS INC.) opi. 26 June 2000, epd. 4 December 1998 (entire document)	1-25
P, X P, Y	Nature Medicine, 5(11), November 1999, 'New DNA enzyme targeting Egr-1 mRNA inhibits vascular smooth muscle proliferation and regrowth after injury', F.S. Santiago et.al., pages 1264-1269 (entire document)	1, 2, 5-7, 9-13, 25 8, 14-21
A	Progress in Nucleic Acid Research & Molecular Biology, 50, 1995, 'Early-Growth Response Protein 1 (Egr-1: Prototype of a Zinc-finger Family of Transcription Factors', A. Gashler and V. P. Sukhatme, pages 191-224 (entire document)	1-25

INTERNATIONAL SEARCH REPORT

International application No.
PCT/AU00/01315

Supplemental Box
(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box No: B

Keywords used

WPAT; Chemical Abstracts - (EGR()1 or NGFI()A or ZIF368 or KROX24 or TIS8 or early growth response) AND (cancer or carcinoma or tumor or tumour or malignant or proliferation or angiogenesis or antisense or ribozyme or DNA()enzyme or DNAzyme or antibod+ or anti()bod+ or antagonist#)

Medline - (early()growth()response()factor()1 or EGR()1 or NGFI()A or ZIF368 or KROX24 or TIS8) AND (cancer or carcinoma or tumor or tumour or malignant or prolif? or angiogen? or antisense or ribozyme or DNA()enzyme or DNAzyme or antibod? or anti()bod? or decreas? or reduc? or bind?)

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/AU00/01315

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
AU A	20865/97	AU B	707943	WO	97/32979		
		EP	934404	CA	2248350		
AU A	44487/00	WO	00/45771				
AU A	21444/00	WO	00/34302	US	6008048		
US	5807718	AU	45950/96	AU B	710747	BR	9510003
		CA	2205382	CN	1173207	EP	792375
		FI	972333	HU	77576	NO	972483
		WO	96/17086	US	5807718		

END OF ANNEX